Adrenergic Receptor Gene Expression in Bovine Leukocytes and Effects of Adrenergic Agonists on Neutrophil and Eosinophil Function

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ABSTRACT

Mammalian responses to stressors that alter homeostasis are mediated by the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes. The SAM axis releases catecholamines, which include the neurotransmitters epinephrine (E) and norepinephrine (NE). The α- and β-adrenergic receptors (ARs) are encoded by 6 α- and 3 β-AR genes and these ARs mediate interactions between the stress hormones E and NE and immune cells. However, AR gene expression and function have been studied to a limited extent in bovine immune cells. Furthermore, a thorough survey has never been published in any species to determine which of the 9 AR genes are expressed in blood leukocytes and if there are significant differences in AR gene expression when comparing among leukocyte lineages.

In this study, AR gene expression was quantified in bovine leukocytes isolated from whole blood, peripheral blood mononuclear cells (PBMCs), polymorphonuclear cells (PMNs), and purified T cells, B cells, monocytes, innate lymphoid cells, neutrophils and eosinophils. While transcript abundance for the β2-, α2A-, and α1A-AR genes tended to be greatest in leukocytes isolated from bovine whole blood, marked differences in AR gene expression were observed when leukocytes were separated into individual lineages. Variation in AR gene expression among leukocyte lineages provided the first evidence that individual bovine leukocyte lineages may also differ in their responses to E and NE. Adrenergic receptor gene expression was also quantified in whole blood leukocytes following maternal separation (weaning) and transportation of suckling beef calves. These stressors were associated with significant increases in transcript abundance for the β1-, β2, β3-, and α2A-AR genes in blood leukocytes at different time points throughout the 28-day post-weaning and transportation period.

Thus, the capacity of the immune system to respond to E and NE may increase significantly when animals respond to stressful changes in their environment.

Following confirmation that AR genes were expressed in bovine leukocytes, PMNs were chosen for further analysis of AR gene expression and function. I developed and validated an appropriate flow cytometric method to specifically identify neutrophils and eosinophils and then flow cytometry was used to analyze the response of resting and activated PMN to adrenergic agonists. Eosinophils were identified as autofluorescence high and CD44 high cells, while neutrophils were characterized as autofluorescence low and CD44 low. PMNs are frequently assumed to be primarily neutrophils with a small proportion of eosinophils. However, I observed, depending on the individual animal and time of year, eosinophils comprised 1.8 - 29.4% of isolated PMN populations. Subsequent analysis of neutrophils and eosinophils confirmed each population responded differently to co-stimulation with opsonized zymosan and IFNγ and displayed significantly different responses to adrenergic agonists.

Short-term treatment with E, NE, phenylephrine (an α1-AR agonist), dexmedetomidine (an α2-AR agonist), and isoproterenol (a β-AR agonist) modified neutrophil and eosinophil expression of intracellular reactive oxygen species, CD11b, L-selectin, CD16 and CD44. Analysis of these markers revealed significant differences in the response of these two PMN subpopulations to these adrenergic agonists. Resting neutrophils demonstrated an L-selectin “shedding” response to E and NE, consistent with a phenotype which may lead to decreased neutrophil exit from, and increased marginated neutrophil entry, into circulation. At the same time, resting neutrophils treated with E and NE increased CD11b and intracellular reactive oxygen species (iROS), consistent with cell activation. This short-term state could be representative of increased neutrophil surveillance in response to stress, leading to an increased inflammatory response to

tissue pathogens, as in the case of bovine respiratory disease. This response is consistent with the short-term neutrophilia and leukocyte activation that has been observed in response to multiple stressors such as weaning and transportation in cattle. Similar responses were observed in activated neutrophils in response to NE.

My research also demonstrated a potential role for eosinophils during a stress response, as eosinophils responded to NE with an activation phenotype (L-selectin high, CD11b high, CD44 high, CD16 high). This eosinophil activation may augment short-term tissue invasion by eosinophils following stimulation with agonists such as NE. Interestingly, synthetic agonists targeting individual AR families (phenylephrine, dexmedetomidine, and isoproterenol) induced some responses that were similar to those observed with E and NE. However, these agonists consistently decreased iROS in both neutrophils and eosinophils. Further research is required to determine why physiological and synthetic AR agonists had different effects on iROS.

Collectively, my analyses of AR gene expression provided evidence that the sympathetic-adrenal-medullary (SAM) axis may interact with all the leukocyte subpopulations examined. Further work is required to determine how this interaction may alter the function of individual leukocyte lineages or subpopulations. My analysis of AR function in PMNs provided evidence both neutrophils and eosinophils express functional α1-, α2- and β-AR, but these two PMN subpopulations differ significantly in their response to adrenergic agonists. However, adrenergic agonists consistently increased iROS and altered expression of surface adhesion molecules by resting neutrophils and eosinophils. Therefore, an acute stress response with increased release of catecholamines could rapidly increase the capacity of neutrophils and eosinophils to respond to infection or tissue damage.

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α1-ARs	alpha 1 adrenergic receptors
α1A-ARs	alpha 1A adrenergic receptors
α1B-ARs	alpha 1B adrenergic receptors
α1D-ARs	alpha 1D adrenergic receptors
α2-ARs	alpha 2 adrenergic receptors
α2A-ARs	alpha 2A adrenergic receptors
α2B-ARs	alpha 2B adrenergic receptors
α2C-ARs	alpha 2C adrenergic receptors
ADAM17	A disintegrin and metalloprotease 17, or tumor necrosis factor α converting enzyme (TACE)
ADHD	attention deficit hyperactivity disorder
AIP	atypical interstitial pneumonia
AMs	alveolar macrophages
ANOVA	analysis of variance
APC	Allophycocyanin
ARs	adrenergic receptors
β-ARs	beta-adrenergic receptors
β1-ARs	beta-1 adrenergic receptors
β2-ARs	beta-2 adrenergic receptors
β3-ARs	beta-3 adrenergic receptors
BHV-1	Bovine-Herpesvirus 1
BLAST	Basic Local Alignment Search Tool
BoZ	bovine serum opsonized zymosan
BRD	Bovine Respiratory Disease
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CD11b	cluster of differentiation 11b
CD16	cluster of differentiation 16
CD44	cluster of differentiation 44
cDNA	complementary deoxyribonucleic acid
CRE	cyclic adenosine monophosphate response element
CR3	complement receptor 3; CD11b
Cq	cycle of quantitation
DAG	1,2-diacyl-glycerol (product of processing of phosphatidylinositol-4,5-biphosphate)
DCFDA	dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
E	epinephrine
eGFP	enhanced green fluorescent protein
ERK 1/2	extracellular signal-regulated protein kinases 1 and 2
EU	European Union
FBS	fetal bovine serum

FcγRIII	fragment crystallizable gamma receptor III
FDA	Food and Drug Administration
FL1	fluorescent channel 1
FL2	fluorescent channel 2
FL3	fluorescent channel 3
FL4	fluorescent channel 4
FITC	fluorescein isothiocyanate
fMLP	n-formyl-methionine-leucyl–phenyalanine
FSC	forward scatter or size of cells as detected by flow cytometry
G_i	Gα subunit inhibitory protein
GM-CSF	granulocyte macrophage colony stimulating factor
GRE	glucocorticoid response element
G_s	Gα subunit stimulatory protein
G_o	Gα subunit other protein
G_q	Gα subunit queer protein
HBSS	Hank's Buffered Saline Solution
HPA	hypothalamic-pituitary-adrenal
ICAM-1	Intercellular Adhesion Molecule-1
IL-1β	interleukin-1β
IL-2Rα	interleukin-2 receptor alpha chain
IL-5	interleukin-5
IL-6	interleukin-6
IL-10	interleukin-10
IL-12	interleukin-12
IL-18	interleukin-18
ILCs	Innate Lymphoid Cells or Natural Killer cells
IFNγ	interferon gamma
IgG1	immunoglobulin G1
IgG2-Fc	immunoglobulin G2-fragment crystallizable receptor
IP_3	Inositol 1,4,5 triphosphate (product of processing of phosphatidylinositol-4,5-biphosphate)
iROS	intracellular reactive oxygen species
LPS	lipopolysaccharide
L-selectin	leukocyte selectin, also known as CD62L
Ly6C	lymphocyte antigen 6C
mAbs	monoclonal antibodies
NCBI	National Center for Biotechnology Information
NE	norepinephrine
NETosis	release of neutrophil extracellular traps (NETs)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
NO	nitric oxide
NRC	National Research Council
PAF	Platelet activating factor
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline

PBSA	calcium and magnesium free phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PerCP/Cy5.5	peridinin chlorophyll-A protein conjugated to cyanin 5.5
PKA	protein kinase A
PLA_2	phospholipase A2
PLC	phospholipase C
PMA	phorbol myristate acetate
PMNs	polymorphonuclear cells
PLA2	Phospholipase A2
PLC	Phospholipase C
rBoIFNγ	recombinant bovine interferon gamma
ROS	reactive oxygen species
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reactions
SAM	sympathetic-adrenal-medullary
sLe^x	Sialyl $Lewis^x$ antigen
SNS	sympathetic nervous system
SOCE	store operated calcium entry
SSC	side scatter or complexity of cells as detected by flow cytometry
Th17	T helper cell 17
TGFβ1	transforming growth factor β1
TNFα	tumor necrosis factor α
TLR	toll like receptor
TLR4	toll like receptor 4

1.0 INTRODUCTION

Bovine Respiratory Disease (BRD) remains a major animal health problem despite decades of vaccination and aggressive use of antimicrobials (Miles, 2009). The persistence of BRD as a clinical problem may be the result of several factors, including new emerging viral and bacterial pathogens (Taylor *et al.*, 2010). Antibiotics provide a helpful therapeutic approach for the control of the bacterial component of BRD, both as metaphylactic and therapeutic treatments (Ball *et al.*, 2019). However, societal pressure is increasing to reduce antibiotic usage due to increasing antibiotic resistance detected in bovine respiratory pathogens (Stanford *et al.*, 2020). The suggestion has been made that a more effective strategy to control BRD may be to develop therapies which better control host responses to BRD pathogens (Miles, 2009), especially since many of the respiratory bacterial pathogens are part of the normal upper respiratory tract microbiota of cattle (Lima *et al.*, 2016; Klima *et al.*, 2019).

The association between BRD and stressful events such as transportation and weaning has been well defined, hence the moniker “shipping fever” (Taylor *et al.*, 2010; Hodgson *et al.*, 2012). However, how stress increases vulnerability to infection and death from BRD is less well defined. Some of the adverse effects of cortisol, of the hypothalamic-pituitary-adrenal (HPA) axis, on host immune responses have been explored (Kadmiel and Cidlowski, 2013; Earley, Buckham, Sporer and Gupta, 2017). However, there has been- less exploration of the impact of the sympathetic-adrenal-medullary (SAM) axis, which produces epinephrine (E) and norepinephrine (NE). Both E and NE may bind to 9 different adrenergic receptors (ARs), which in turn stimulate individual but overlapping biochemical pathways (Elenkov *et al.*, 2000; Lorton and Bellinger, 2015; Schena and Caplan, 2019; BioRad.com, 2020a, 2020c, 2020b) (Table 3.1, Table 3.2, Table 3.3).

The 9 ARs are important drug targets in humans and animals. Targeting individual subtypes of these receptors has led to therapeutics for a great number of conditions including asthma, anaphylaxis, ADHD, heart conditions, and glaucoma (Barnes, 1993; Kemp *et al*., 2008; Giovannitti, Thoms and Crawford, 2015; Noguchi *et al*., 2015; Farzam and Lakhkar, 2019a; Ueda *et al*., 2020). Targeting the β2-AR subtype is also routinely used to increase lean muscle mass in cattle (Lean, Thompson and Dunshea, 2014; Loneragan, Thomson and Scott, 2014; Thomson *et al.*, 2015; Buntyn *et al.*, 2016; Frese *et al.*, 2016).

The ARs on bovine immune cells have received minimal attention. Exploration of the impact of these receptors on immune function during BRD also remains poorly defined but could yield important biological insights. These insights may reveal mechanisms by which stress increases vulnerability to respiratory disease and guide the discovery of effective immunotherapeutics to prevent BRD. Furthermore, exploration of the interactions between stress, ARs, and BRD may provide an important model for human medicine. There is already evidence that immunotherapy in the form of adrenergic drugs can reduce BRD morbidity when used as a prophylactic. A study in the 1980s in Czechoslovakia indicated that pre-treatment of calves with a drug that blocks β-adrenergic receptors reduced -BRD illness by approximately half (Rašková *et al.*, 1987). Minimal follow-up on this study has been completed, and further research on the impact of adrenergic receptors on the bovine immune system holds promise for improving the prevention and treatment of BRD and possibly other inflammatory conditions.

2.0 RESEARCH HYPOTHESES AND OBJECTIVES

For the purpose of my research project, I addressed the following hypotheses by asking specific questions and used several methods to validate my results:

1. Hypothesis: Differences in adrenergic receptor subtype expression occur among bovine blood leukocyte subpopulations with dominant expression of the β2-AR gene.
    - Determine which ARs are expressed on individual high-speed cell sorted bovine blood leukocytes lineages using Real-time RT-qPCR
2. Hypothesis: Adrenergic receptor gene expression in blood leukocytes will be altered following the stress of maternal separation and transportation.
    - Quantify AR gene expression in blood leukocytes using real-time RT-qPCR analysis and compare AR gene expression in suckling calves versus calves subjected to maternal separation, with or without transportation
3. Hypothesis: Adrenergic receptor agonists will suppress activation of stimulated neutrophils and eosinophils but have no effect on resting neutrophils and eosinophils. Furthermore, these two PMN subpopulations will differ in their responses to adrenergic agonists.
    - Use endogenous and synthetic AR agonists and flow cytometry to quantitatively analyze a variety of *ex vivo* cellular responses by both resting and activated bovine neutrophils and eosinophils

3.0 LITERATURE REVIEW

3.1 Stress: HPA vs. SAM axis

Psychological stress in the body works through two axes: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis (Thornton and Andersen, 2006). Stimulation of the HPA axis leads to the release of cortisol from the adrenal cortex. Stimulation of the SAM axis leads to the release of catecholamines from the adrenal medulla. These catecholamines include the neurotransmitters epinephrine (E), norepinephrine (NE), and dopamine (Thornton and Andersen, 2006; William Tank and Lee Wong, 2014). For the purposes of my project, I will be focusing on E and NE and their effects on bovine immune cells. Epinephrine is synthesized from NE by the enzyme phenylethanolamine n-methyltransferase, and NE is synthesized from dopamine by the enzyme dopamine β-hydroxylase (Végh *et al.*, 2016). Both these enzymes are abundant in the adrenal medulla (Rush and Geffen, 1980; Ziegler *et al.*, 2002) but minimal evidence exists for significant expression of these enzymes in immune cells (Levite, 2012).

3.2 Bovine Respiratory Disease, Neutrophils, and Stress

Bovine Respiratory Disease (BRD) is a major health problem in the beef industry, compromising 40-50% of the industry's infectious disease mortality (Edwards, 2010). This results in significant economic losses. BRD is associated with several primary viral infections, such as Bovine-Herpesvirus 1 (BHV-1), and several different secondary bacterial infections, such as *Mannheimia haemolytica (M. haemolytica*). Secondary bacterial infections frequently cause a necrotizing pro-inflammatory response in the lung that is dependent upon neutrophil

recruitment to the lung (McGuire and Babiuk, 1984; Slocombe *et al.*, 1985; Breider *et al.*, 1988; Caswell *et al.*, 1998; Li *et al.*, 2002).

The greatest BRD morbidity and mortality commonly occurs following maternal separation (weaning) and transportation of 5-6 month old beef calves (Mormede *et al.*, 1982; Chirase *et al.*, 2004; Aich, Potter and Griebel, 2009; Edwards, 2010; Taylor *et al.*, 2010). This increased BRD incidence has been associated with stress-induced changes in immune function which increases mortality following a combined viral and bacterial respiratory infection (Hodgson *et al.*, 2012). Since BRD represents a disease complex involving multiple viruses and bacteria, stress-induced changes in immune function may represent an important therapeutic target for reducing BRD morbidity and mortality.

3.3 Eosinophils in Respiratory Diseases of Cattle

Atypical interstitial pneumonia (AIP) occasionally occurs in cattle. This condition is characterized by emphysema and diffuse edema in the lung (Doster, 2010). Eosinophilia in the lung is occasionally but unreliably associated with AIP. Some potential causes include exposure to L-tryptophan, 4-ipomeanol, perilla ketone (from purple mint), turnip, rape, or kale tops, or sweet potatoes that have gone moldy. In some cases, bacterial or viral infections may also contribute to AIP. However, AIP has not been associated with an allergic reaction (Doster, 2010). In Europe, this condition is also called “fog fever”, and is often observed after cattle begin grazing pastures with lush regrowth of grass following hay cutting in moist “fog lands” (Doster, 2010). AIP is a condition still being researched and investigated to better define etiology.

Certain parasites have also been shown to elicit respiratory distress in cattle, provoking an eosinophil response in the lung. Infection with the bovine lungworm, *Dictyocaulus*

viviparous, often creates severe respiratory illness and interstitial pneumonia in cattle, raising both lung and blood eosinophil counts. Eosinophil activity in the lung has also been found to contribute to the pathogenesis of the disease (Gånheim, Höglund and Waller, 2004; Glasgow, 2019). Migrating larvae of both *Ascaris suum* and *Ascaris lumbricoides*, which also commonly infect pigs and humans, have also been found to cause interstitial pneumonia in cattle. Eosinophils may also contribute to the pathogenesis of the lung reaction to these parasites as well. (Doster, 2010; Shapiro, Peregrine and Caswell, 2017). Thus, eosinophils can also be involved in bovine respiratory diseases.

3.4 Adrenergic Receptors and Stress

Numerous studies have shown that cortisol, of the HPA axis, has a range of immunosuppressive effects on immune cells (Kadmiel and Cidlowski, 2013). However, research on E and NE, of the sympathetic nervous system (SNS), has produced conflicting results, with either inhibition (Elenkov *et al.*, 2000; Trabold, Gruber and Fröhlich, 2007; Gosain, Gamelli and DiPietro, 2009a; Scanzano *et al.*, 2015; Margaryan *et al.*, 2017) or enhancement of immune responses (Elenkov *et al.*, 1995; Kim *et al.*, 2014; Margaryan *et al.*, 2017). I hypothesized that the stress hormones E and NE may alter the function of bovine immune cells in ways that may contribute to the increased susceptibility to fatal respiratory disease observed following maternal separation and transportation of suckling beef calves. However, there is very little information regarding the expression of ARs on bovine immune cells and this lack of knowledge makes it difficult to postulate how stress may alter bovine immune function.

Adrenergic receptors bind E and NE, and each receptor can mediate unique cellular responses. For example, ARs are expressed by myocardial cells in the heart and binding of

circulating E and NE facilitates increased contractility in response to stress. ARs are also expressed in adipose tissue to promote lipolysis and release of sugar into the bloodstream upon binding of catecholamines (Taylor, 2007; Alhayek and Preuss, 2018). ARs are also expressed on immune cells but their function may vary depending on the specific type of immune cell and the AR subtype expressed by the cell. There has been limited characterization of AR expression by bovine immune cells (LaBranche, Ehrich and Eyre, 2010). Furthermore, gaps remain as well in our understanding of AR expression and function in human immune cells. For example, α-AR expression has not been determined for human eosinophils. Thus, further research is required to determine the role ARs play in regulating immune cell function in many different species.

In cattle, a drug known to block β-AR signaling, metipranolol, was shown to reduce BRD by ~50% in 1-2 week old weaned and transported calves (Raškova *et al.*, 1987). Thus, insight into the effects of ARs on bovine immune cell function may provide important insights into new strategies for BRD prevention. Furthermore, drugs which influence AR signaling may provide an effective intervention that reduces industry reliance on antimicrobials.

3.5 Adrenergic Receptor Expression and Function

The ARs serve important functions in facilitating cellular responses to stress in many different organs (Table 3.1, Table 3.2, Table 3.3). Interestingly, these receptors are also expressed on immune cells. Here, their function is complex, and varies depending on the type of immune cell and the type of AR expressed (Table 3.4, Table 3.5, Table 3.6). Differential expression of ARs on different cell types may provide clues as to a cell's response to stress (Lorton and Bellinger, 2015). Thus, the study of ARs on bovine immune cells may provide further insight into modulation of bovine immune system functions by E and NE.

3.5.1 α1-Adrenergic Receptors: Expression and Function in Non-Lymphoid Tissues

The three α1-ARs are encoded by the *ADRA1A, ADRA1B,* and *ADRA1D* genes. α1-ARs are coupled to the G_q or G_{11} proteins which stimulate Phospholipase C, occasionally Phospholipase A2 (Piascik and Perez, 2001; Chew and Ong, 2016), the L-type calcium channel, and protein kinase C (Barnes, 1993; Lorton and Bellinger, 2015; BioRad.com, 2020a, 2020c) (Table 3.1). Ultimately, this signaling leads to stimulation of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (BioRad.com, 2020a, 2020c). Human α1-ARs are expressed at high levels in the heart, brain, and smooth muscle such as that found in the urinary tract (Kavelaars, 2002; Uberti, Hall and Minneman, 2003; Karabacak *et al.*, 2013). The α1A- and α1D-ARs are highly expressed in the human brain, urinary tract, and blood vessels (Karabacak *et al.*, 2013; Scanzano and Cosentino, 2015). The α1A-ARs tend to localize to renal and caudal arterial smooth muscle, and are involved in regulating blood pressure (Piascik and Perez, 2001). The α1B-AR has a particularly high expression in the human spleen and is also highly expressed in the human kidney (Perez, 2006). These receptors do not regulate blood pressure (Piascik and Perez, 2001). Similar to α1A-ARs, α1D-ARs are also highly expressed in the human brain, urinary tract, and blood vessels (Karabacak *et al.*, 2013; Scanzano and Cosentino, 2015). However, the α1D-ARs tend to localize to the aorta and iliac, femoral and superior mesenteric arteries, also regulating blood pressure (Piascik and Perez, 2001). Thus, despite similarities among the α1-AR subtypes, they are differentially expressed and play distinct roles in different tissues.

3.5.2 α1-Adrenergic Receptors: Expression and Function on Immune Cells

The α1-ARs, when stimulated, have primarily a pro-inflammatory effect within the immune system (Grisanti *et al*., 2011) (Table 3.4). For example, pre-treatment of mice with

prazosin, an α1-AR antagonist, was associated with reduced TNFα production following LPS injection (Sugino *et al.*, 2009). It has also been observed that an LPS treated human monocyte cell line increased IL-1β secretion when exposed to phenylephrine, an α1-AR agonist (Grisanti, Perez and Porter, 2011). Increased complement component C2 synthesis in human monocytes was also observed following phenylephrine stimulation. This effect was reversed by the α1-AR antagonist prazosin (Grisanti, Perez and Porter, 2011). Expression of α1-ARs has been detected during early human leukocyte development in the bone marrow and thymus. Following maturation and leukocyte entry into blood, α1B-AR expression tends to disappear from human leukocytes. For instance, dendritic cells (DCs) down-regulate α1B-AR expression as they mature (Kavelaars, 2002). Functional α1-ARs have, however, been identified on lymphocytes and most innate immune cells (Barnes, Carson and Nair, 2015; Scanzano and Cosentino, 2015). The α1-ARs primarily bind NE (Barnes, Carson and Nair, 2015) and the effects of α1-ARs on immune cell function warrants further investigation.

3.5.3 α2-Adrenergic Receptors: Expression and Function in Non-Lymphoid Tissues

The three α2-ARs are encoded by the *ADRA2A, ADRA2B,* and *ADRA2C* genes. The α2-ARs are coupled to the G_i or G_o proteins, which inhibit adenylyl cyclase and consequently inhibit Protein Kinase A but stimulate Phospholipase C, activating intracellular calcium release (Scanzano and Cosentino, 2015; BioRad.com, 2020b) (Table 3.2). Ultimately, α2-AR signaling leads to stimulation of the ERK1/2 pathway (BioRad.com, 2020b). The α2-ARs primarily bind E, but can also bind NE (Barnes, Carson and Nair, 2015; Lorton and Bellinger, 2015). The α2-ARs are highly expressed in brain, renal epithelial cells, and skin arteriole or vein smooth muscle cells (Saunders and Limbird, 1999). The α2A-ARs are also highly expressed in the rat brain stem, human spleen, and human kidney (Saunders and Limbird, 1999; Scanzano and Cosentino,

2015). Expression of the α2A-AR has also been reported for the bovine retina and brain (Venkataraman, Duda and Sharma, 1997; Inderwies *et al*., 2003). In humans, α2B-AR transcript has been studied, and demonstrated to have high expression in the kidney and liver (Perez, 2006). Transcript for the α2C-AR is also highly expressed in the human brain and kidney (American Society of Anesthesiologists. *et al.*, 1994; Scanzano and Cosentino, 2015) and in the brain is localized to the midbrain and thalamus (Saunders and Limbird, 1999). The α2-ARs play important roles in neuron differentiation and function. The α2B-ARs induce a short term, salt-induced, vasoconstrictive hypertensive response, and α2A- and α2C-ARs can induce longer term systemic hypotension through an inhibition of sympathetic outflow and NE release (Philipp, Brede and Hein, 2002). Consequently, drugs which stimulate α2-ARs are commonly used as sedatives in humans (ex: dexmedetomidine) and animals (ex: xylazine) (Amouzadeh, 1991; Giovannitti, Thoms and Crawford, 2015; Wang *et al.*, 2019a). Further study of α2-ARs in other cell types may reveal additional biological effects.

3.5.4 α2-Adrenergic Receptors: Expression and Function on Immune Cells

Stimulation of α2A-ARs has a variety of effects on immune cells (Table 3.5). The α2A-ARs primarily bind E but can also bind NE (Barnes, Carson and Nair, 2015; Lorton and Bellinger, 2015). Human PMNs exposed to *Escherichia coli. ex vivo* decrease nitric oxide and nitric oxide synthase production when treated with dexmedetomidine (α2-AR agonist) (Chen *et al.*, 2016). Clonidine, another α2-AR agonist, inhibited influenza virus replication both *in vitro* and in mice (Matsui *et al.*, 2018). Given that drugs which stimulate α2A-ARs are commonly used as sedatives in humans, exploration of their impacts on the immune system is now being further explored. For instance, a systematic literature review and meta-analysis of stress and immune effects of dexmedetomidine on humans was recently completed. Correlations between

administration of dexmedetomidine as an anaesthetic and an inhibition of E, NE, cortisol, and blood glucose were observed. These physiological changes may contribute to the sedative effect. Dexmedetomidine was also associated with an anti-inflammatory state. This state included decreased release of TNFα, IL-6, C-reactive protein, and increased IL-10 production. Changes in blood leukocyte populations were also noted. These changes included increased numbers of B cells, NK cells and CD4 T cells but decreased numbers of CD8 T cells circulating in blood (Wang *et al.*, 2019a). It is important to note that the effects associated with dexmedetomidine may reflect a direct interaction with immune cells or an indirect effect, possibly through an inhibition of stress hormones such as NE (Philipp, Brede and Hein, 2002). Human PMNs have been observed to express mRNA for the α2A and α2C-AR but not for the α2B-AR (Scanzano *et al.*, 2015). It is clear from these studies that α2-ARs impacts on the immune system, either direct or indirect, warrant further exploration.

3.5.5 β-Adrenergic Receptors: Expression and Function in Non-Lymphoid Tissues

The three β-ARs are encoded by the *ADRB1, ADRB2,* and *ADRB3* genes. These receptors are usually coupled to the G_s proteins, stimulating adenylate cyclase, producing cyclic AMP (cAMP) and stimulating protein kinase A (PKA) (Elenkov *et al.*, 2000; Lorton and Bellinger, 2015; Scanzano and Cosentino, 2015) (Table 3.3). Occasionally, β-ARs can couple with the G_i protein, consequently inhibiting this same signaling pathway. β2-ARs primarily bind E but β1-ARs and β3-ARs can bind both E and NE with a greater affinity for E (Barnes, Carson and Nair, 2015; Scanzano and Cosentino, 2015). A high level of β1-AR expression is routinely observed in human heart while high levels of β2-AR expression are observed on the smooth muscle of bronchioles (Taylor and Bristow, 2004; Cazzola, Calzetta and Matera, 2011). The β3-AR is abundant in human adipose tissue (Larsen *et al*., 2002; Scanzano and Cosentino, 2015). Bovine

β-AR transcript has been observed in 12 tissues, including muscle, subcutaneous fat, heart, liver, lung, kidney, large and small intestine, omasum, reticulum, and rumen, and spleen (Mei *et al.*, 2018). Transcript has also been detected in the bovine mammary gland (Inderwies *et al.*, 2003).

Although β- and α-ARs are relatively well-conserved among vertebrate species, their distribution and primary function can vary (Zavala et al. 2017; Mei et al. 2018; Taylor and Bristow 2004; LaBranche, Ehrich and Eyre 2010). For instance, β3-AR predominates in human adipose tissue and is associated with lipolysis (Taylor and Bristow, 2004) but primarily β2-AR is observed in bovine adipose tissue (Mei *et al.*, 2018). Thus, species-specific neuromodulation of immune cell function may become apparent when investigating the distribution and modulation of ARs within bovine immune cells. Overall, there has been limited study of AR expression and function in cattle, particularly on immune cells (Labranche, 2005; Mei *et al.*, 2018).

3.5.6 β-Adrenergic Receptors: Expression and Function on Immune Cells

The β-ARs are expressed on a wide range of human immune cells. Maisel et al. (1990) reported β-AR abundance with a decreasing order of NK cells > CD14 monocytes > CD8 T cells > B cells > CD4 T cells. Among the β-ARs, β2-AR is often expressed at the highest level on human immune cells, particularly ILCs and lymphocytes (Maisel *et al.*, 1990; Lorton and Bellinger, 2015). Expression of β1- and β2-ARs, was detected with the radiolabeled antagonist CGP-12177 and was similar on leukocytes from male and female cattle. Bovine PBMCs also expressed a higher level of these receptors relative to bovine PMNs (Labranche, Ehrich and Eyre, 2010).

Ligand binding by β-ARs, such as the β2-AR expressed on immune cells, appears to primarily suppress cell responses (Gu and Seidel, 1996; Lamas, Martínez and Marti, 2003;

Emeny, Gao and Lawrence, 2007; Grisanti *et al*., 2010; LaBranche, Ehrich and Eyre, 2010; Lorton and Bellinger, 2015) (Table 3.6). For example, Gu and Seidel (1996) observed decreased production of TNFα and reactive oxygen species (ROS) following treatment of bovine alveolar macrophages and differentiated human macrophages with salbutamol (β2-AR agonist) and isoproterenol (non-selective β-AR agonist) prior to stimulation with lipopolysaccharide (LPS) and zymosan, respectively. This is consistent with studies using human and murine monocytes and macrophages, which reported decreased release of the pro-inflammatory mediators TNFα, IL-12, and nitric oxide (NO), following isoproterenol (β-AR agonist) stimulation (Szelenyi *et al*., 2006a). Of interest, Verhoeckx et al. (2005) demonstrated that following LPS injection into rats, zilpaterol, a β2-AR agonist commonly used to increase muscle mass in steers and heifers (Buntyn *et al.*, 2016), reduced TNFα production. This was validated both *in vivo* and *in vitro* (Verhoeckx *et al.*, 2006). Labranche et al. (2010) demonstrated induction of cAMP and decreased ROS production following stimulation of β-ARs on bovine PMNs (LaBranche, Ehrich and Eyre, 2010). The β-ARs remain the best studied group of ARs in immune cells, including bovine immune cells.

Table 3.1: Signaling pathways used by individual α1-ARs.

Adrenergic Receptor and ligand	G proteins associated	Adenylyl Cyclase Effect	Phospholipase Associated	Produce	Protein Kinase Effect	Relevant Pathway
α1A primarily NE (Barnes, Carson and Nair, 2015)	G-alpha-q G-alpha-11 G-alpha-i4 (BioRad.com, 2020a)	None (BioRad.com, 2020a)	PLC-beta-1[1] (BioRad.com, 2020a) PLA2[2] (Piascik and Perez, 2001; Chew and Ong, 2016)	IP_3[3] DAG[4] ↓ Ca^{2+} Release (BioRad.com, 2020a)	PKC Activation (BioRad.com, 2020a)	ERK1/2 (BioRad.com, 2020a)
α1B* primarily NE (Barnes, Carson and Nair, 2015)	G-alpha-q G-alpha-11 G-alpha-14 G-alpha-15 G-alpha-o (BioRad.com, 2020a)	None (BioRad.com, 2020a)	PLC-beta-1 PLC-delta-1 (BioRad.com, 2020a) PLA2 (Piascik and Perez, 2001; Chew and Ong, 2016)	IP_3 DAG ↓ Ca^{2+} Release (BioRad.com, 2020a)	PKC Activation (BioRad.com, 2020a)	ERK1/2 (BioRad.com, 2020a)
α1D* primarilys NE (Barnes, Carson and Nair, 2015)	G-alpha-q G-alpha-11 (BioRad.com, 2020a)	None (BioRad.com, 2020a)	PLC-beta-1 PLC-delta-1 (BioRad.com, 2020a) PLA2 (Piascik and Perez, 2001; Chew and Ong, 2016)	IP_3 DAG ↓ Ca^{2+} Release (BioRad.com, 2020a)	PKC Activation (BioRad.com, 2020a)	ERK1/2 (BioRad.com, 2020a)

*associated with transglutaminase 2 (BioRad.com, 2020a)

1. PLC = Phospholipase C
2. PLA2 = Phospholipase A2
3. IP_3 = Inositol 1,4,5 triphosphate (product of processing of phosphatidylinositol-4,5-biphosphate)
4. DAG = 1,2-diacyl-glycerol (product of processing of phosphatidylinositol-4,5-biphosphate)

Table 3.2: Signaling pathways used by individual α2-ARs.

Adrenergic Receptor and ligand	G proteins associated	Adenylyl Cyclase Effect	Phospholipase Associated	Produce	Protein Kinase Effect	Relevant Pathway
α2A (extracellular) (Philipp, Brede and Hein, 2002) primarily E, but also NE (Barnes, Carson and Nair, 2015; Lorton and Bellinger, 2015)	G-alpha-i2 G-alpha-i3 G-alpha-i1 G-alpha-o (BioRad.com, 2020b)	Inhibits adenylyl cyclase ↓ Decreases cAMP	PLC-beta-3[1] PLC-beta-2 (BioRad.com, 2020b)	IP_3[2] DAG[3] ↓ Ca^{2+} Release (BioRad.com, 2020b)	PKA Inhibition (BioRad.com, 2020b)	ERK1/2 (BioRad.com, 2020b)
α2B (extracellular) (Philipp, Brede and Hein, 2002) primarily E, but also NE (Barnes, Carson and Nair, 2015; Lorton and Bellinger, 2015)	G-alpha-i2 (BioRad.com, 2020b)	Inhibits adenylyl cyclase ↓ Decreases cAMP	PLC-beta-3 PLC-beta-2 (BioRad.com, 2020b)	IP_3 DAG ↓ Ca^{2+} Release (BioRad.com, 2020b)	PKA Inhibition (BioRad.com, 2020b)	ERK1/2 (BioRad.com, 2020b)
α2C (*intracellular* and extracellular) (Philipp, Brede and Hein, 2002) primarily E, but also NE (Barnes, Carson and Nair, 2015; Lorton and Bellinger, 2015)	G-alpha-i family (BioRad.com, 2020b)	Inhibits adenylyl cyclase ↓ Decreases cAMP	PLC-beta-3 PLC-beta-2 (BioRad.com, 2020b)	IP_3 DAG ↓ Ca^{2+} Release (BioRad.com, 2020b)	PKA Inhibition (BioRad.com, 2020b)	ERK1/2 (BioRad.com, 2020b)

1. PLC = Phospholipase C
2. IP_3 = Inositol 1,4,5 triphosphate (product of processing of phosphatidylinositol-4,5-biphosphate)
3. DAG = 1,2-diacyl-glycerol (product of processing of phosphatidylinositol-4,5-biphosphate)

Table 3.3: Signaling pathways used by individual β-ARs.

Adrenergic Receptor and ligand	G proteins associated	Adenylyl Cyclase Effect	Phospholipase Associated	Produce	Protein Kinase Effect	Relevant Pathway
β1 binds both E and NE, but E > NE (Barnes, Carson and Nair, 2015; Scanzano and Cosentino, 2015)	G-alpha-s (Alhayek and Preuss, 2018)	Activates adenylyl cyclase ↓ Increases cAMP (Alhayek and Preuss, 2018)	None	cAMP Ca^{2+} influx (Alhayek and Preuss, 2018)	PKA Activation (Alhayek and Preuss, 2018)	cAMP-PKA axis (Alhayek and Preuss, 2018)
β2 primarily E (Barnes, Carson and Nair, 2015; Scanzano and Cosentino, 2015)	G-alpha-s (canonical) G-alpha-i (non-canonical) (Lorton and Bellinger, 2015)	Activates adenylyl cyclase ↓ Increases cAMP (Lorton and Bellinger, 2015)	None (Lorton and Bellinger, 2015)	cAMP (Lorton and Bellinger, 2015)	PKA Activation (Lorton and Bellinger, 2015)	cAMP-PKA axis When coupled to G-alpha-I→ ERK1/2 (Lorton and Bellinger, 2015)
β3 resistant to desensitization (Schena and Caplan, 2019) binds both E and NE, but E > NE (Barnes, Carson and Nair, 2015; Scanzano and Cosentino, 2015)	G-alpha-s G-alpha-i (Schena and Caplan, 2019)	Activates adenylyl cyclase ↓ Increases cAMP (Schena and Caplan, 2019)	None	cAMP (Schena and Caplan, 2019) When coupled to G-alpha-i→ Nitric Oxide (Schena and Caplan, 2019)	PKA Activation (Schena and Caplan, 2019)	cAMP-PKA axis When coupled to G-alpha-i→ ERK1/2 (Schena and Caplan, 2019)

Table 3.4: Selected examples of functional cell responses following α1-AR stimulation.

Adrenergic Receptor	Cellular Responses	Immune Responses	Cell Types Studied
α1A[1]	Renal and caudal arterial smooth muscle contraction (Piascik and Perez, 2001), hypertrophic growth of neonatal cardiomyocytes (Piascik and Perez, 2001), smooth muscle contraction, proliferation, protein synthesis (BioRad.com, 2020a, 2020c)	Increased complement synthesis on monocytes (Grisanti, Perez and Porter, 2011), Increase in IL-1β secretion from LPS treated monocytes (Grisanti, Perez and Porter, 2011), Increase of histamine release in murine mast cells (Moroni *et al.*, 1977), Increased systemic levels of IL-10 in LPS treated animals (Dong *et al.*, 2002; Sugino *et al.*, 2009) Increases in dendritic cell differentiation and migration (Maestroni, 2000)	Murine Arterial smooth muscle Human and other species' cardiomyocytes Human Monocytes Murine Dendritic cells Murine Mast cells Human PBMCs Murine Dendritic cells
α1B*	Hypertrophic growth of cardiomyocytes (Piascik and Perez, 2001), cell proliferation, smooth muscle contraction, protein synthesis (BioRad.com, 2020a, 2020c)		
α1D*	Hypertrophic growth of aortic smooth muscle cells in rats, smooth muscle contraction of aorta and femoral, iliac, and superior mesenteric arteries (Piascik and Perez, 2001) smooth muscle contraction, protein synthesis (BioRad.com, 2020a, 2020c)		

Table 3.5: Selected examples of functional cell responses following α2-AR stimulation.

Adrenergic Receptor	Cellular Responses	Immune Responses	Cell Types Studied
α2A[1] (extracellular) (Philipp, Brede and Hein, 2002)	Cell proliferation (Cai *et al.*, 2014; BioRad.com, 2020b), neuron differentiation, causes longer-term hypotension, hypothermia (Philipp, Brede and Hein, 2002)	Inhibits murine langerhan cell migration (Maestroni, 2000), Induces resistance to *Mycobacterium avium* in mouse macrophages by increasing peroxynitrite production (Weatherby, Zwilling and Lafuse, 2003),	Murine Neurons Porcine and Rat proximal tubule cells Murine Langerhans cells Mouse macrophages Human blood Human PMNs
α2B (extracellular) (Philipp, Brede and Hein, 2002)	Cell proliferation (Cai *et al.*, 2014; BioRad.com, 2020b), neuron differentiation. causes short-term hypertension (Philipp, Brede and Hein, 2002)	Inhibits influenza virus replication in mice (Matsui *et al.*, 2018) , Inhibits blood levels of inflammatory cytokines such TNFα, IL-6. Increases numbers of cell groups such as NK cells, B cells, CD8 T cells (Wang *et al*., 2019b),	
α2C (*intracellular* and extracellular) (Philipp, Brede and Hein, 2002)	Cell proliferation (Cai *et al.*, 2014; BioRad.com, 2020b), neuron differentiation. causes short-term hypertension (Philipp, Brede and Hein, 2002)	Inhibits systemic levels of cytokines in humans such as eotaxin, interleukin-18, interleukin-2Rα, stem cell factor, stem cell growth factor and vascular endothelial growth factor (Kallioinen *et al*., 2019), Decreased nitric oxide and nitric oxide synthase release in *E. coli* treated human PMNs (Chen *et al*., 2016)	

Table 3.6: Selected examples of functional cell responses following β-AR stimulation.

Adrenergic Receptor	Cellular Responses	Immune Responses	Cell Types Studied
β1	Increases heart rate (Taylor, 2007), (Alhayek and Preuss, 2018), decreases plasma renin and increases renal vascular resistance (Bakris, Hart and Ritz, 2006), thermogenesis and lipolysis in adipocytes (Soloveva *et al.*, 1997) (Casteilla *et al.*, 1994)	Increased IL-1β production in LPS-stimulated human monocytes (Grisanti *et al.*, 2010), Increases TNFα in LPS treated mice (Elenkov *et al.*, 1995), impairs cell mediated response to Listeria in mice (Emeny, Gao and Lawrence, 2007)	Human Heart cells Mouse, Bovine Adipose tissue Human monocytes Human kidney
β2	Relaxes bronchial smooth muscle (Barnes, 1993; Grove, McFarlane and Lipworth, 1995; Cazzola, Calzetta and Matera, 2011), Increases glycogenolysis and gluconeogenesis (Erraji-Benchekroun *et al.*, 2005), Increases lean muscle mass in cattle and humans, particularly fast twitch muscles. Non-steroidal anabolic drugs (Sato *et al.*, 2011), Increases systolic arterial blood pressure (Du *et al.*, 2000)	Gs coupled transduction generally immunosuppressive, inhibits NF-κB signalling. β2-AR can desensitize with chronic stimulation leading to systemic inflammation (Elenkov *et al.*, 2000; Lorton and Bellinger, 2015) decreases TNFα, ROS release from bovine macrophages (Gu and Seidel, 1996), inhibits NETosis in human neutrophils (Marino *et al.*, 2018), inhibits ROS production in bovine PMNs (LaBranche, Ehrich and Eyre, 2010)	Human Bronchial smooth muscle cells Mouse Heart cells Rat Liver cells Bovine, Human Muscle cells
β3 resistant to desensitization (Schena and Caplan, 2019)	Lipolysis, thermogenesis (Lowell and Flier, 1997; Moreno-Aliaga *et al.*, 2002; Schena and Caplan, 2019), increased bladder capacity (relaxes detrusor smooth muscle). Minimal cardiovascular effects (Sacco and Bientinesi, 2012; Di Salvo *et al.*, 2017), retinal cell growth and migration (Schena and Caplan, 2019), Increased (Gs) or decreased (Gi) heart contractility (Schena and Caplan, 2019)	Increases CD4+ lymphocytes in mouse spleen (Lamas, Martínez and Marti, 2003) Inhibit ROS production in human myometrial macrophages (Hadi *et al.*, 2017)	Human, Murine, Adipose tissue Mouse Spleen Human Retina Human, Monkey, Rat Bladder Human Heart Human Myometrial macrophages

[1]A lack of drugs and antibodies with α1-AR subtype and α2-AR subtype selectivity has limited research in this area. However, a number of drugs target either α1-AR or α2-ARs.
ROS = reactive oxygen species

3.6 Adrenergic Receptor Gene Organization, Regulation, and Cross-Regulation

Genes for the nine bovine ARs are distributed across many different chromosomes and do not share a common promoter (National Center for Biotechnology Information, 2021). However, stress hormones can regulate AR gene expression. Hadcock, Wang and Malbon (1989) observed dexamethasone, a synthetic glucocorticoid receptor agonist, upregulated *ADRB2* transcription. Furthermore, they observed β-AR agonists could destabilize β-AR mRNA. In combination, glucocorticoids and β-AR agonists were shown to counteract each other's effects on *ADRB2* transcription. In 1993, Hadcock and Malbon, in a literature review, noted AR cross-regulation could occur. For example, increases in cellular cAMP stimulated by β-AR activity could increase the expression of α2A- and α1-AR mRNA. It was observed by Sakaue and Hoffman (1991) that an element promoting responsiveness to cAMP was located in the *ADRA2A* gene's promoter region. This was later identified as the cAMP response element or CRE, and is also present in the *ADRB2* gene (Hadcock and Malbon, 1993). More research has identified a glucocorticoid response element, or GRE, in the noncoding 5' region of the *ADRB2* gene (Hadcock and Malbon, 1993). In 1994, Kiely et al. observed that dexamethasone downregulated *ADRB1* mRNA and upregulated *ADRB2* mRNA. They observed that both genes have a GRE. In monocytes, it has been observed that both glucocorticoids and β2-agonists up-regulate *ADRA1B* and *ADRA1D* mRNA but not *ADRA1A* mRNA (Kavelaars, 2002). Thus, both glucocorticoids and catecholamines can influence adrenergic receptor expression.

3.7 Adrenergic Receptor Modulation of Granulocyte Responses

3.7.1 Human and Murine Neutrophils

Several studies have analyzed the *in vitro* and *in vivo* effects of adrenergic drugs on human and murine neutrophils. Trabold, Gruber and Fröhlich (2007) examined neutrophil responses to E and NE using fMLP stimulated whole blood cultures. Neutrophils were identified through flow cytometric gating on cell size and esterase activity. They observed that E and NE prevented fMLP-induced increases in CD11b expression and decreases in cell surface L-selectin. In 2009, Gosain, Gamelli and DiPietro examined the effect of NE on neutrophil recruitment to wounds created in mice. They collected leukocytes retained by sponges placed in the wound and used flow cytometric gating to identify neutrophils. They observed that pharmacological doses of NE reduced phagocytosis of fluorescent *E. coli* by neutrophils isolated from wounds 5 days after treatment. Both α- and β-ARs were observed to contribute to this effect. Thus, there is evidence that E and NE can modulate both adhesion molecule expression and phagocytosis by neutrophils.

Further studies demonstrated that E can influence PMN trafficking to wounds and that adrenergic drugs can influence multiple aspects of PMN function. In 2014, Kim et al. examined the effect of E on immune cell involvement in wound healing in transgenic eGFP-lys mice. In eGFP-lys mice, greater than 95% of eGFP High cells are PMNs. Treatment with E doubled the number of Ly6C+/CD11b+ PMNs in mouse wounds, slowing wound repair. PMN trafficking was primarily mediated by the pro-inflammatory action of β2-ARs on macrophages and IL-6 was an important mediator of this effect. In 2015, Scanzano et al. tested the effects of E and NE on isolated human PMNs. They observed that E, NE, and the β2-AR agonist isoproterenol reduced

the fMLP stimulated ROS response, reduced cell migration, and decreased expression of CD11b/CD18. Resting PMNs did not respond to adrenergic drugs. The β-AR played an important role in mediating the changes in ROS production. However, transcripts for all ARs, except α2B, were detected in human PMNs. When PMNs were stimulated with fMLP, β-AR transcript expression was observed to increase. The β-ARs appeared to a play an important role in modulating PMN function in these studies.

The role of adrenergic drugs in modulating different aspects of PMN function, including cytokine production and neutrophil NET formation, was also examined. Margaryan et al. in 2017 used flow cytometry to specifically analyze human PMNs in whole blood cultures. Pharmacological concentrations of E had a slight pro-inflammatory effect (increased CD11b and IL-8) in resting PMNs. In contrast, E had an immunosuppressive effect (decreased CD11b and CD18, IL-8, IL-1β, and MCP-1) in LPS-stimulated PMNs. Marino et al. (2018) studied in greater detail the function of β-ARs in human PMNs. They observed that stimulation of β2-ARs inhibited neutrophil extracellular trap (NET) formation in human neutrophils. This response was measured by ROS and elastase production, and light and fluorescent microscopic evaluation. Activation of the β2-AR also inhibited PMN migration, as measured with a Boyden chamber assay. PMN inhibition by β2-AR agonists was most evident when cells were stimulated with LPS, fMLP, or IL-8. Taken together, there is increasing evidence that ARs, particularly β-ARs, play an important role in modulating PMN function.

3.7.2 Human and Murine Eosinophils

Several studies have examined the effect of adrenergic drugs on human and murine eosinophils as well. Humphreys and Raab (1950) demonstrated that epinephrine injection into humans caused a 61% decrease in blood eosinophils. Norepinephrine was also tested, but only

caused a 10% reduction in blood eosinophils. Collection of blood samples from medical students revealed a similar decrease (48%) in blood eosinophils prior to exams, suggesting this was part of a stress response. Koch Weser (1968) reported a similar 62% decrease in circulating blood eosinophils following epinephrine injection and this effect was blocked with propranolol, a β-AR antagonist. By itself, propranolol caused a 27.8% increase in blood eosinophils. Furthermore, α-adrenergic blockers were unable to prevent the eosinopenia caused by epinephrine. Collectively, these results support the conclusion that the eosinopenia induced by epinephrine was mediated by β-ARs.

Research on AR function in eosinophils has been limited primarily to their role in asthma. Treatment of asthma often includes the use of inhaled β2-agonists, and eosinophils are considered an important element of the asthmatic response (Noguchi *et al.*, 2015). For example, in the interest of exploring a possible relationship between β2-ARs, eosinophils, and asthma, Yukawa *et al.* (1990) used radioligand binding to quantify β2-ARs on both human and guinea pig eosinophils. Eosinophils were isolated from patients with eosinophilia using Percoll gradients, achieving 80% or greater purity. Compared to neutrophils, the eosinophils appeared to have a higher density of β2 receptors (4300 receptors/eosinophil as compared to 900-1800/neutrophil). Eosinophils also had higher affinity β2 receptors (as measured with salbutamol) than neutrophils (Kd ~28.7 pM for eosinophils compared to Kd ~1nM for neutrophils). Eosinophils were stimulated with opsonized zymosan or phorbol myristate acetate (PMA) and both stimuli elicited a superoxide anion and peroxidase response. However, exposure to salbutamol (albuterol), a β2 adrenergic agonist, failed to modify this reaction. A cAMP response was, however, elicited indicating β2-ARs were functional on human eosinophils

(Yukawa *et al.*, 1990). Thus, the role of β2-ARs on eosinophils and their potential relationship to asthma is still being investigated.

Bates et al. (1994) reported that blood epinephrine levels and eosinophil counts were significant predictors of nocturnal asthma. Normally, blood epinephrine levels begin decreasing in the late afternoon and are at their lowest concentration around 4 AM before increasing until wakefulness. It was observed that patients with lower than normal epinephrine levels around 4 PM and 10 PM were at high risk of nocturnal asthma, and an episode often occurred around 4 AM, when epinephrine was lowest. A higher blood eosinophil count over the course of 3 days was also predictive of nocturnal asthma in patients. However, the authors speculated eosinophilia may be representative of the overall level of inflammation in patients, rather than a mechanism mediating an episode of nocturnal asthma. An even more sensitive predictor of nocturnal asthma was the blood eosinophil to epinephrine ratio. Cortisol was measured, and also displayed standard rhythm. However, cortisol concentration did not correlate significantly with nocturnal asthma. Thus, the authors recommended that the eosinophil to epinephrine ratio be used as a sensitive predictor of nocturnal asthma episodes.

Noguchi et al. (2015) also examined the function of β2-ARs on eosinophils enriched to 98% purity with magnetic cell sorting. Purified eosinophils were stimulated with interleukin-5 (IL-5), leukotriene D4 (LTD4) or IFNγ inducible protein of 10 kD (IP-10). Treatment with salbutamol, a standard β2-AR agonist, was found to not affect stimulated eosinophils. However, formoterol, a potent β2-AR agonist, altered multiple functions of activated eosinophils. This included suppression of eosinophil adhesion to ICAM-1 and suppression of both superoxide anion and eosinophil-derived neurotoxin production. Resting eosinophils demonstrated no detectable reaction to this agonist.

Kainuma et al. (2017) reported that when eosinophils and bronchial epithelial cells were co-cultured, bronchial epithelial cells transitioned to inflammatory mesenchymal cells, potentially contributing to fibrosis of the airway in asthma. These mesenchymal cells express a more pro-inflammatory phenotype. For example, they secrete greater levels of the Th17 cytokine transforming growth factor β_1 (TGFβ_1) and granulocyte-macrophage-colony stimulating factor (GM-CSF). Exposure of eosinophils to a β2-AR agonist (procaterol) prior to co-culture with bronchial epithelial cells prevented this epithelial cell transition effect.

Few studies have analyzed α-AR expression or function in eosinophils (Scanzano and Cosentino, 2015). Liu et al. (2020) analyzed eosinophils isolated from the conjunctiva of mice following exposure to the short ragweed allergen. Murine conjunctival eosinophils were confirmed to express transcript for the α1A-AR gene *ADRA1A*, but transcript for all other ARs was undetectable. Restraint stress significantly increased blood NE levels and eosinophil recruitment to the conjunctiva. The use of α1A-AR agonists A 61603 hydrobromide and tunsulosin hydrochloride helped confirm that the α1A-AR was responsible for increased eosinophil recruitment to the murine conjunctiva during restraint stress.

3.7.4 Bovine PMNs

There is also evidence that bovine PMNs express functional ARs. Labranche et al. (2005) used radioligand binding studies to confirm β1-AR and β2-AR expression on bovine PMNs and PBMCs. Functional β2-AR expression was confirmed using terbutaline, a β2-AR agonist, to inhibit ROS production by activated PMNs. There is evidence that adrenergic agonists modulate other aspects of bovine PMN function as well. Holroyde and Eyre (1976) demonstrated that the histamine response by allergen-sensitized bovine PMNs was increased with low concentrations (10^{-6} – 10^{-5} M) of β-AR agonist and decreased by a higher concentration (10^{-4}-10^{-3} M) of an α1-

AR agonist. To prepare sensitized bovine PMNs, 4–6-week-old calves were injected with horse plasma and PMNs were harvested from bovine blood and exposed to horse plasma antigen *ex vivo* to stimulate histamine release. The β-AR responsible for potentiating the histamine response at lower agonist concentrations was later demonstrated to be β1-AR (Perron and Eyre, 1982). They also reported a biphasic response to isoproterenol (β1- and β2-AR agonist) within a similar dose range. In other species, epinephrine and/or isoproterenol are known to have a suppressive effect on histamine release. New research is demonstrating that cell types such as neutrophils, not just basophils, can produce histamine (Xu et al., 2006; Smuda, Wechsler and Bryce, 2011). Thus, this research may confirm adrenergic modulation of several types of PMNs and bovine PMNs may be unique in their AR expression and function.

A study by Raškova et al. (1987) indicated metipranolol, a β-AR antagonist, reduced BRD incidence by approximately 50%. This prompted a follow-up study to investigate possible mechanisms of action by β-AR antagonists. Given the important role of PMNs in BRD pathogenesis, Henricks et al. (1990) explored the effect of β-AR antagonists on bovine PMNs exposed to *M. haemolytica,* an important BRD pathogen. A key virulence factor for this bacterium is leukotoxin, a secreted protein which reduces the ROS response of bovine PMNs. The β-AR antagonists propranolol and alprenolol blocked inhibition of the ROS response induced by *M. haemolytica* leukotoxin. However, when β1- and β2-AR specific antagonists were evaluated neither reversed the decrease in ROS caused by leukotoxin. This may indicate that inhibition was mediated by the β3-AR. Thus, non-selective β-AR antagonists may block the suppressive immunomodulatory effects of ARs when bovine PMNs are exposed to *M. haemolytica* leukotoxin. These *in vitro* studies confirm that ARs may modulate the function of PMNs, which play an important role in BRD. Further, these data suggest one possible

mechanism by which an AR blocking agent may reduce the effect of stress on BRD (Raškova et al. 1987).

Catecholamines have been shown to alter other aspects of bovine PMN function. Diez-Fraile et al. (2000) examined the effect of several different adrenergic drugs on LPS induction of CD11b, a cell adhesion molecule, expression on bovine PMNs. L-Phenylephrine, an α1-AR agonist, had no effect on CD11b expression, while dexamethasone, a potent glucocorticoid receptor agonist, significantly decreased CD11b expression. Both isoproterenol, a β-AR agonist, and clenbuterol, a β2-AR agonist, decreased CD11b expression. A combination of dexamethasone and isoproterenol had a synergistic effect on decreasing CD11b expression. This study analyzed CD11b expression on bovine PMNs but whole blood was incubated with dexamethasone and AR agonists. Thus, the authors hypothesized that the LPS-induced increase in CD11b expression on PMNs may have been caused indirectly by TNFα released from LPS-stimulated monocytes. This hypothesis was supported by the observation that adding TNFα by itself to blood samples increased CD11b expression on PMNs. Thus, the direct effect of adrenergic drugs on bovine neutrophils and eosinophils remains unknown.

Collectively these studies provide evidence that bovine PMNs express functional ARs, including β1-AR, β2-AR, and possibly α1-ARs, and that individual ARs may mediate distinct PMN responses. However, there are several technical parameters that may impact the biological relevance of the reported observations. First, most analyses were performed using PMNs which consist of neutrophils, eosinophils and a minor population of basophils. It is not known if neutrophils and eosinophils, and basophils express the same ARs and if signaling by ARs is similar among these PMN subpopulations. Furthermore, all the cited studies, with the exception of LaBranche, Ehrich and Eyre (2005), conducted their experiments with cells incubated at 37°C.

LaBranche, Ehrich and Eyre (2005) were the only group to incubate PMNs at 39 °C, the normal body temperature of cattle. Many studies also used adrenergic agonists outside the normal physiological range for cattle (~15 to 150 nM; Buhler *et al*., 1978), as in the studies by LaBranche, Ehrich and Eyre (2005), Holroyde and Eyre (1976), and Diez-Fraile et al. (2000)**.** Although these studies provide indications that bovine PMNs express functional ARs, there are limitations in the published data that leave substantial gaps in our knowledge regarding AR expression and function in bovine PMNs.

3.7.5 Bovine Eosinophils

Few studies have addressed the effect of adrenergic drugs on bovine eosinophils. Based on the marked eosinopenia observed following E injection in humans, a similar study was performed with cattle (Alexander, 1958). However, no significant change in circulating eosinophils was observed following E injection in cattle. The injected E did have a biological effect, however, since there was a marked decrease in milk production. A similar failure to alter circulating eosinophils was also observed when E was injected into sheep (Zarrow *et al.*, 1952) and horses (Alexander and Ash, 1955). However, injecting cattle with adrenocorticotropic hormone (ACTH), a potent inducer of cortisol release, reduced circulating eosinophils by more than 50%. A study analyzing the effects of transportation, also reported that transporting cattle over mountainous terrain reduced blood eosinophils. This effect may have been mediated by cortisol effect since a cortisolemia occurred in the transported group (Ishizaki and Kariya, 2010).

3.8 Response of Bovine Neutrophils to Stress

Neutrophils can vary from 22 to 76% of the leukocytes present in normal bovine blood ((George, Snipes and Lane, 2010) but their frequency can change rapidly during infection or stress. Short term increases in PMN number in blood are observed following abrupt weaning and transportation (Hickey, Drennan and Earley, 2003; Yagi *et al*., 2004; Ishizaki and Kariya, 2010; O'Loughlin *et al*., 2012). Ishizaki et al. (2010) observed a neutrophilia in cattle 4 hours after transportation over mountainous terrain, as well as an increase in the neutrophil to lymphocyte ratio. Yagi et al. (2004) observed neutrophilia at the end of a 4-hour transport that persisted 2 hours later. They also noted a decrease in apoptotic PMNs, indicating an increase in immature PMNs from the marginated pool. O'Loughlin et al. (2012) recorded an increase in blood neutrophils one day after weaning suckling calves. Hickey et al. (2003) also noted a significant increase in blood neutrophils up to 3 days following weaning stress. Thus, both weaning and transport stress are known to alter neutrophil numbers in bovine blood.

PMNs play an important role during BRD and previous literature implicates ARs as one mechanism that may influence the prevalence of respiratory disease (Raškova *et al.*, 1987). However, modulation of bovine PMN function by adrenergic drugs remains an unexplored therapeutic approach to the control of infectious disease. PMNs play a key role in controlling bacterial and fungal infections (Roth and Kaeberle, 1981) but may also contribute to the immune pathology associated with respiratory infections in cattle (Slocombe *et al.*, 1985; Li *et al.*, 2002; Hodgson *et al.*, 2012; Allen and Kurdowska, 2014). Given the important role of PMNs during BRD, and previous literature indicating that ARs play a role in BRD infection (Raškova *et al.*, 1987), modulation of bovine PMN function with adrenergic drugs appears worth further exploration.

3.9 Measures of Neutrophil and Eosinophil Activation

There are numerous studies indicating adrenergic drugs modulate the responses of PMNs following activation with agents such LPS, fMLP, phorbol myristate acetate (PMA), or opsonized zymosan (Haskó *et al.*, 1995a; Gu and Seidel, 1996; Szelenyi *et al.*, 2006; Trabold, Gruber and Fröhlich, 2007; LaBranche, Ehrich and Eyre, 2010; Scanzano *et al.*, 2015). There are fewer reports, however, indicating adrenergic drugs can alter the responses of resting PMNs (Margaryan *et al.*, 2017). Thus, to analyze the effects of adrenergic drugs on bovine PMNs it is important to establish methods that consistently activate bovine PMNs and develop assays that quantitatively measure bovine neutrophil and eosinophil activation. The following sections review several methods used to measure activation in PMNs.

3.9.1 Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) is an important microbicidal response activated in neutrophils during inflammation. This begins with the intracellular generation of O_2-superoxide anions by NADPH oxidase. These anions are converted by myeloperoxidase to hydrogen peroxide (H_2O_2) and H_2O_2 is further transformed by myeloperoxidase to a variety of secondary ROS. These include species such as hypochlorous acid, which is considered a particularly potent ROS. ROS are then directed towards microbes within PMN phagosomes. They can also be released extracellularly as part of DNA based Neutrophil Extracellular Traps (NETs) but excess production and release of ROS can cause tissue damage (Winterbourn, Kettle and Hampton, 2016; Villagra-Blanco *et al.*, 2017).

A comparison of human and bovine PMN responses to activation stimuli revealed bovine serum opsonized zymosan (BoZ) induced a broad range of activation responses in bovine PMNs, including a vigorous ROS response (Brown and Roth, 1991). For this reason, BoZ stimulation is frequently chosen as a strategy to “activate” bovine neutrophils. Un-opsonized zymosan

induction of ROS production in bovine PMNs is dependent on CD11b and Store Operated Calcium Entry (SOCE) (Conejeros *et al*., 2011). IgG2-Fc receptors have also been implicated in mediating the additional PMN activation caused by serum opsonization of zymosan (L. Leino and Paape, 1993).

Combining recombinant bovine interferon gamma (rBoIFNγ) with BoZ was found to further increase the ROS response in bovine PMNs (Sample and Czuprynski, 1990). In my studies, it was observed a lower dose of BoZ can be combined with rBoIFNγ to amplify PMN responses to BoZ and reduce cell clumping. Stimulation with rBoIFNγ has been shown to reduce bovine PMN migration under agarose and increase bovine PMN cell-mediated toxicity against chicken erythrocytes. This has been shown to occur in both an antibody-dependent and antibody-independent manner (Steinbeck, Roth and Kaeberle, 1986). By itself, however, rBoIFNγ did not alter ROS production (Steinbeck, Roth and Kaeberle, 1986; Sample and Czuprynski, 1990). Thus, rBoIFNγ is generally considered a potent bovine PMN activation factor (Steinbeck, Roth and Kaeberle, 1986).

Human eosinophils, enriched to greater than 80% purity with Percoll gradients, responded to opsonized zymosan and phorbol myristate acetate (PMA) with increased levels of superoxide anion and eosinophil peroxidase (Yukawa *et al.* 1990). In asthma patients, eosinophil release of ROS has been found to be integral to the process of eosinophil extracellular trap (EET) formation and is an important contributor to the pathology of asthma in the lung and bronchioles (Silveira et al., 2019). There has been limited study of ROS in bovine eosinophils. However, in isolated bovine eosinophils and neutrophils, it was observed that PMA had a much stronger effect on the ROS response, as measured by chemiluminescence, in eosinophils versus

neutrophils (Freiburghaus, Jörg and Müller, 1991). Overall, ROS generation can be used as an important measure of neutrophil and eosinophil activation.

3.9.2 CD11b

CD11b, also known as alpha-M integrin, is part of the β2 integrin CD11b/CD18 complex and plays an important role in neutrophil adhesion to vasculature following stimulation by chemoattractants (*C*oxon *et al*., 1996; Ince, Weber and Scheiermann, 2019b). It is also involved in Fc receptor mediated PMN degranulation (Tang *et al*. 1997) and acts as complement receptor 3 (CR3; Berends *et al.*, 1993). Thus, CD11b plays an important role in PMN responses to and subsequent amplification of lung inflammatory responses. CD11b has also been shown to play an important role in apoptosis and turnover of extravasated neutrophils (Coxon *et al.*, 1996). During systemic inflammatory conditions, such as those created by low endotoxin doses, a CD11b high neutrophil subpopulation emerges, characteristic of an "aged" neutrophil phenotype (Casanova-Acebes *et al.*, 2013). Increased CD11b expression is considered an indicator of an activated neutrophil with a pro-inflammatory phenotype (Cassetta *et al*., 2019). On eosinophils, CD11b has also been found to play an important role in recognition of fungi such as *Alternaria alternata*, an environmental fungus implicated in triggering asthma (Yoon et al., 2008). A strong correlation has also been found between high CD11b expression on blood eosinophils and histamine hyper-responsiveness (In't Veen *et al.*, 1998). Thus, increased expression of CD11b can be a marker of activation on both neutrophils and eosinophils.

3.9.3 CD16

CD16, also known as FcγRIII, is a low-affinity Fc receptor associated with PMN deployment of Ca^{2+}, degranulation, oxidative burst, and phagocytosis (Huizinga *et al.*, 1990;

Kimberly *et al.*, 1990). High CD16 expression is associated with human neutrophil extracellular trap (NET) formation (Millrud *et al.*, 2017) and is considered a marker of neutrophil maturation and activation (Brandau *et al.*, 2011). Increased CD16 expression on PMNs is observed in patients with bacterial and viral respiratory infections and allergies, as well as patients given low doses of endotoxin (Pillay *et al.*, 2012). Thus, CD16 may provide an important marker for adrenergic modulation of PMN function. A high level of CD16 expression on eosinophils has been characterized as an "allergic" phenotype. In healthy humans eosinophils have been shown to express low to no CD16. However, when patients with allergic asthma or allergic rhinitis were exposed to increasing doses of allergen, a CD16 high population of eosinophils emerged (Davoine *et al.*, 2002). Thus, increased expression of this Fc receptor may be a marker of activation in both neutrophils and eosinophils.

3.9.4 CD44

CD44 is a surface receptor involved in adhesion to hyaluronan in the extracellular matrix. It was observed that CD44 deficient mice had 84% higher neutrophil recruitment to the lung during *E. coli* induced pneumonia. This increased recruitment was attributed to reduced CD44 binding to hyaluronan of the extracellular matrix which normally restricts neutrophil motility (Wang *et al.*, 2002). When an allergic response was induced in mice, administration of anti-CD44 antibodies reduced eosinophil migration to the lung, allergen hyper-responsiveness, production of cytokines such as IL-4 and IL-5, and hyaluronan production in the lungs. Thus, there is evidence that CD44 plays an important role in regulating the eosinophil allergic airway response, as well as the allergic response in general (Katoh *et al.*, 2003). On neutrophils, lower expression of CD44 may be a marker of activation, while a different CD44 response may be observed in activated eosinophils.

3.9.5 L-Selectin

L-selectin, is an adhesion, homing, and signaling molecule present on most leukocytes. It is generally considered a "tethering/rolling" receptor. Upon cell activation, a process denoted as L-selectin "shedding" from cells occurs (Ivetic, Green and Hart, 2019) A process of "mechanical" L-selectin shedding while rolling on endothelium is also described in PMNs (Lee *et al.*, 2007). A subset of activated, "mature" neutrophils with low L-selectin expression are observed at sites of inflammation (Casanova-Acebes *et al.*, 2013; Tak *et al.*, 2017). Trabold, Gruber and Fröhlich (2007) observed that E and NE prevented L-selectin shedding by fMLP stimulated human PMNs. In a study examining unchallenged moderate to severe atopic asthmatics, they found no difference in L-selectin expression on blood and sputum eosinophils relative to non-atopic controls (In't Veen *et al.*, 1998). However, in one study of allergic asthmatics, it was found that eosinophils in bronchoalveolar (BAL) fluid displayed an L-selectin low phenotype 4 hours after allergen challenge (Mengelers *et al.*, 1993). Thus, changes in L-selectin expression on eosinophils may be integral to the allergic asthmatic response. An L-selectin "shedding" response would be expected on activated neutrophils, and possibly activated eosinophils as well.

3.10 Adrenergic Receptors as Drug Targets

3.10.1 Common Pharmacological Uses

Adrenergic receptors are commonly used as drug targets; there are several important FDA approved uses of AR agonists and antagonists. These include Epipens, which contain epinephrine and consequently stimulate multiple ARs (Farzam and Lakhkar, 2019a). Epipens help reverse the large decrease in blood pressure that comes with anaphylactic shock, reduce airway constriction, and help reduce the release of inflammatory mediators (Kemp *et al.*, 2008).

Agonists for the α2-AR such as clonidine and dexmedetomidine are regularly used as anaesthetics, for the treatment for attention deficit hyperactivity disorder, and as treatment for hypertension (Giovannitti, Thoms and Crawford, 2015). General β-AR antagonists such as propranolol are occasionally used as treatment for anxiety disorders and performance anxiety (Steenen *et al.*, 2016). In asthma, β2-agonists such as salbutamol (albuterol) can be used in "puffers" to relax smooth muscle of the bronchioles and prevent the release of histamine from mast cells, improving breathing (Barnes, 1993; Noguchi *et al.*, 2015; Ueda *et al.*, 2020).

In cattle and pigs, an important use of adrenergic drugs is the use of β2-agonists such as zilpaterol (trade name: Zilmax) and ractopamine to increase lean muscle mass (Lean, Thompson and Dunshea, 2014). Zilpaterol was temporarily removed from the market in the US and Canada by Merck Animal Health following anecdotal concerns of animals arriving at slaughterhouses with sloughed hooves. (Loneragan, Thomson and Scott, 2014; Thomson *et al.*, 2015). Following further research, there was no clear evidence establishing a link between β2-agonists and this phenomenon and zilpaterol was reintroduced to the market (Buntyn *et al.*, 2016; Frese *et al.*, 2016). However, its use is still banned in the EU, China, and Russia. Interestingly, β2-agonists are also used as performance-enhancing drugs in humans, and are thus considered a "doping" agent in professional sports (Bizec, 2017). Thus, controversy remains over the use of β2-agonists to increase lean muscle mass.

3.10.2 Adrenergic Agonist and Antagonist Specificity

Adrenergic drugs have varying degrees of specificity for adrenergic receptors. The β-adrenergic receptors, which have received the most study, have the broadest array of available agonists and antagonists. Isoproterenol is commonly used as a non-selective β-AR agonist in experimental studies and therapeutically (Shimizu et al., 1996; Szymanski and Davinder,

2021). Several drugs are considered selective agonists for individual β-AR subtypes. For the study of β1-AR activity, for example, there is the agonist dobutamine. This drug is sometimes used for short-term support in heart failure. However, dobutamine can also bind to a lesser extent to both the β2- and α1-ARs, so is not completely selective (Mraz and Rorabaugh, 2007). Specific β2-AR agonists include albuterol (salbutamol) and formoterol. (Faulds, Hollingshead and Goa, 1991). More recently, drugs that selectively target the β3-AR have been developed. This includes mirabegron, which is used therapeutically to treat overactive bladder. At higher than recommended doses (ex: 200 mg), some off-target effects on β1 and β2-ARs appear (Kashyap and Tyagi, 2013), but at therapeutic doses mirabegron is considered a selective β3-AR agonist. The availability of drugs that are agonists for β-ARs in general and for individual β-AR subtypes has facilitated a much better understanding of β-AR function.

Although drugs are available that selectively target α1-ARs in general, there are few drugs which selectively target individual α1-AR subtypes (Chen and Minneman, 2005). Phenylephrine, for example, targets the entire α1-AR family (Richards, Lopez and Maani, 2019) and has become a standard reagent when studying the function of α1-ARs in general (Jensen, O'Connell and Simpson, 2011). A drug named A 61603 is a more selective agonist for just the α1A-AR (Knepper et al., 1995) and BMY 7378 is an antagonist that more selectively binds α1D-AR (Goetz et al., 1995) but also binds 5-HT1A receptors (Stubbs, Connor and Feniuk, 1991). Identifying drugs that specifically target the α1B-AR has proven more difficult (Chen and Minneman, 2005). Thus, research on the specific functions of individual α1-AR subtypes remains an emerging area.

Drugs that target individual α2-AR subtypes have also not been identified, limiting the characterization of α2-AR subtype function. When targeting the family of α2-ARs, clonidine is a

commonly used agonist (Farzam and Lakhkar, 2019b). However, clonidine also binds with lower affinity to all α1-AR subtypes (a ratio of 220:1 for α2-ARs vs. α1-ARs) (Giovannitti, Thoms and Crawford, 2015). Furthermore, in competition binding experiments clonidine was found to have only a 22-fold binding preference for α2A-AR versus the 5-HT1A (serotonin) receptor (Newman-Tancredi et al., 1998). In contrast, dexmedetomidine exhibits a binding specificity of 1620:1 for α2-ARs versus α1-ARs (Giovannitti, Thoms and Crawford, 2015) and 91-fold greater binding with α2A-AR versus 5-HT1A receptors. Dexmedetomidine is therefore considered a more selective α2-AR agonist than clonidine (Newman-Tancredi et al., 1997). More recently, a novel antagonist, AGN-209419, was identified that is specific for the α2B-AR. This drug has been used to characterize the expression and function of this AR in the brain (Luhrs et al., 2016), but has otherwise been used little to study α2B-AR function. Selective agonists or antagonists for α2-AR subtypes are still mostly unavailable, and most studies have used drugs that target the α2-AR family.

3.11 Knowledge Gaps and Potential Relevance

To date, there has been limited study of ARs in bovine immune cells. Particularly, it remains largely unknown which bovine immune cells express individual ARs and what functions individual ARs may regulate within each immune cell type (LaBranche, Ehrich and Eyre, 2010). Gaps also remain in our understanding of the expression and function of ARs on human immune cells. Some potential benefits of studying ARs in cattle include a greater understanding of how stress modulates bovine immune function, a greater understanding of how β-agonist drugs such as zilpaterol may affect bovine health, and potential augmentation of human and bovine drug crossovers for therapeutic control of tissue damage caused by excessive inflammation.

4.0 Methods and Materials

4.1 Blood Collection

The phlebotomy protocol (AUP #20170015) was reviewed and approved by the University of Saskatchewan University Animal Care Committee following Canadian Council on Animal Care gquidelines. Blood was collected from the jugular vein using 60 ml syringes (Becton Dickinson, Franklin Lakes, NJ) and an 18 g PrecisionGlide hypodermic needle (Becton Dickinson) with 0.3% EDTA (Sigma Aldrich, St. Louis, MO) used as an anticoagulant.

3.2 Isolation of Blood Leukocytes

Blood leukocytes were isolated by lysis of red blood cells (RBCs). Briefly, a 3 mL aliquot of bovine blood was diluted with 12 mL 1X RBC lysis buffer (0.17 M NH4Cl, 10 mM KHCO3, and 0.11 mM EDTA, pH 7.3). Cells were centrifuged for 8 minutes at 311X g to pellet leukocytes. Lysis buffer was discarded and the leukocyte pellet re-suspended in 1 mL Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Ltd., Paisley, UK) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences Inc., Lenexa, KS). Leukocyte concentration was determined with a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Indianapolis, IN). Leukocytes were diluted to a final concentration of 10 X 10^6 cells/ml and aliquots of 10 X 10^6 cells transferred to 2 mL cryopreservation tubes (Corning, Inc. New York, NY). Cells were centrifuged at 350 X g for 5 minutes, supernatant was discarded, and cell pellets snap-frozen in liquid Nitrogen or re-suspended in TRIzol (ThermoFisher Scientific, Waltham, MA). Cell pellets were stored at -80 C prior to RNA extraction.

4.3 Peripheral Blood Mononuclear Cell Isolation

Peripheral Blood mononuclear cells (PBMCs) were isolated as follows. Briefly, blood was centrifuged at 1400g for 20 minutes without braking at room temperature. Following centrifugation, the buffy coat layer at the interface between plasma and the red blood cells was collected. The buffy coat was added to 20 mL calcium- and magnesium-free phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4; pH 7.3) containing 0.1% EDTA and layered over a 15 mL column of 60% Percoll (density = 1.13 g/mL; GE Healthcare BioSciences, Uppsala, Sweden). Samples were centrifuged at 2000X g for 20 minutes without braking. The buffy coat was once again collected and re-suspended in PBS containing 0.1% EDTA before centrifuging at 311X g for 8 minutes. The pelleted PBMCs were re-suspended in PBS and centrifuged at 150X g for 8 minutes before being suspending cells at an appropriate concentration in PBS for staining with monoclonal antibodies (mAbs) in preparation for flow cytometry or high-speed cell sorting.

4.4 PMN Isolation

Briefly, 50 ml of blood was centrifuged at 1400 X g for 20 minutes without braking at 4°C. Following centrifugation, plasma and the buffy coat layer with ~15% of the underlying red blood cells were removed. The remaining red blood cells and PMNs were re-suspended with PBS containing 0.1% EDTA and centrifuged again at 1400 X g for 20 minutes without braking. The PBS and red blood cells exceeding the 16 mL mark in a 50 mL tube were removed. The remaining blood was gently pipetted to re-suspend PMNs and 4 ml aliquots were distributed among four 50 mL tubes before adding 46 mL 1X RBC Lysis Buffer (0.17 M NH4Cl, 10 mM KHCO3, and 0.11 mM EDTA, pH 7.3) to each tube. Cells were centrifuged at 325 X g for 6 minutes, and the lysis buffer was decanted. The PMN cell pellet was re-suspended in PBS

containing 0.1% EDTA before centrifuging at 325 X g for 5 minutes. PMNs were then washed twice with PBS.

4.5 RNA Extraction

RNA was extracted from blood leukocytes using a combined TRIzol/RNEasy Mini Kit extraction method. Frozen cell pellets were lysed with 1 mL TRIzol reagent (ThermoFisher Scientific) and 200 uL chloroform (Sigma Aldrich) was added to each sample. Samples were shaken for 15 seconds then incubated at room temperature for 2-3 minutes before centrifuging for 15 minutes at 13,282 X g. Following centrifugation, the aqueous phase was removed and an equal amount of 70% ethanol (Greenfield Global, Brampton, ON) added. Following this step, samples were added to the silica columns provided in the RNEasy Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) and processed according to the manufacturer's instructions.

For samples with low expected RNA yields, primarily those with less than 1×10^6 cells or PMNs, the RNA elution step was repeated once, and RNA was concentrated using the Savant Speed-Vac DNA concentrator SC110A (Thermo Electron Co., Milford, MA).

4.6 RNA quality assessment

RNA concentration and quality was determined using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

4.7 cDNA Synthesis

Synthesis of complementary DNA (cDNA) from RNA template was done following the manufacturer's instructions for the Quantitect Reverse Transcription Kit (Qiagen). A 30 minute cDNA synthesis incubation step (42°C) was used to denature RNA secondary structure. Briefly, 500 ng RNA was diluted in 6 μL UltraPure DNAse/RNAse-Free Distilled Water (Invitrogen, Carlsbad, CA) and 1 μL of 7x gDNA wipe-out buffer was added to remove genomic DNA. The

GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA) was used to incubate this mixture for 2 min at 42°C. Following incubation, 3 μL master mix was added to each reaction. The master mix for each reaction consisted of 2 μL 5x Quantiscript RT buffer, 0.5 μL of primer mix, and 0.5 μL reverse transcriptase. Following addition of the master mix, each reaction was incubated for 30 minutes at 42°C, followed by 3 minutes at 95°C.

4.8 Reverse Transcription Quantitative PCR

Reverse transcription quantitative polymerase chain reactions (RT-qPCR) were prepared with 5 μL of 5ng/μL cDNA providing 25 ng cDNA/reaction, and 10 μL of master mix for a total reaction volume of 15 μL. The master mix consisted of 7.5 μL 2X PERFECTA-IQ SYBR Green Supermix (QuantaBio, Beverly, MA), 2.2 μL UltraPure DNAse/RNAse-Free Distilled Water (Invitrogen), and 0.3 μL of 10 μM forward and reverse primer (3 pmoles; Table 4.1). Reactions were run in Hard Shell Low-Profile 96-well semi-skirted, clear shell and clear well PCR plates (BioRad, Hercules, CA). The CFX Connect Real Time System (BioRad) was used to run and quantify real time PCR reactions. Reactions were first run at 95°C for 2 min (once) to activate the hot-start Taq polymerase, then for 40 cycles at 95°C for 15 sec (denature), 60°C for 30 sec (anneal), and 72°C for 30 sec (extend). Following amplification, a melt curve was applied for detection of abnormal products. The melt curve started at 65°C, and the temperature held for 10 seconds before increasing by 1°C. This pattern was repeated to a temperature of 95°C. Results were visualized using the CFX Manager/Maestro software and corrections for primer efficiency were included in Cq value calculations.

4.9 Primer Design and Validation

Primers were designed for the 9 known bovine AR genes (Table 4.1). CloneManager software was used to select for *AR* gene amplicons between 80 to 150 bp, and these primer sets

were screened against the bovine (*Bos taurus*) genome on the National Center for Biotechnology Information's (NCBI's) website. This software was used to select three primers for each gene which had a low likelihood of amplifying false products. These primers were tested to see if they amplified a product of the correct size and tested for efficiency. Primers which had high efficiency and amplified a product of the correct size had products sent for sequencing at the National Research Council (NRC). The β – Actin gene was chosen as a reference gene for analysis as it had previously been validated as a housekeeping gene in bovine blood (Robinson, Sutherland and Sutherland, 2007; González-Cano *et al.*, 2014). The reference genes YWAHZ and GAPDH were also tested in combination with this reference gene, but the most consistent results were found to be obtained just using β-Actin. The forward and reverse β – Actin primers used were those published by González-Cano et al (2014). Oligonucleotides were ordered from Invitrogen by Thermo Fisher Scientific.

4.10 Validation of Amplicon Size

Confirmation that amplicons of the predicted size were generated with each primer pair was obtained by visualizing PCR products on a 1.5% UltraPure Agarose gel (Invitrogen). RNA for this analysis was isolated from bovine tissues predicted to have high AR transcript abundance and all primers generated a single visible product.

4.11 Primer Efficiencies

Primer products were ligated into plasmids and plasmids were amplified in *E. coli*. Plasmids were purified and diluted to the same concentrations. Primer efficiencies were determined by using serial 10-fold dilutions of digested plasmids containing individual ADR gene products and subsequent amplification using qPCR with 2X PERFECTA-IQ SYBR Green

Supermix (QuantaBio). Primer efficiency values were determined using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001).

All primers (Table 4.1) had an amplification efficiency greater than 85%. Selected primers were confirmed to generate a single peak on a melt curve, in addition to amplifying a single visible product on gel electrophoresis.

Table 4.1: Validated Primers for Bovine AR Genes

Gene (Accession number)	Primer Sequence	Product Size (bp)	Primer Efficiency (%)
ADRB1	F 5' – CCTAGCCAACGTGGTGAAGG - 3'	117	90.8
(NM_194266)	R 5' – CTGCGGCAGTAGATGATGGG -3'		
ADRB2	F 5' – TAGCCATCACGTCACCCTTC - 3'	100	93.6
(NM_174231)	R 5' – AAGGAGGTAAGGCCAGACAC -3'		
ADRB3	F 5' – TTCTGCCTCTGCGGGAACAC - 3'	99	91.3
(NM_174232)	R 5' – GCACCACGTTGACCACAAAG -3'		
ADRA1A	F 5' – GATTTCAGGCCCTCAGAAAC – 3'	142	88.5
(NM_174498)	R 5' – GCAGACACTGGATTCTCAAG – 3'		
ADRA1B	F 5' – CCCTTCTATGCCCTCTTCTC – 3'	109	91.7
(NM_001191139)	R 5' – GGTTCTTGGTGGTCCTCTTG – 3'		
ADRA1D	F 5' – CTTCCTGGCAGCCTTCATCC – 3'	144	89.6
(NM_001205684)	R 5' – GGAGCACTGTGGCACTTAGC – 3'		
ADRA2A	F 5' – CATCGGAGTGTTCGTGGTGT – 3'	122	90.9
(NM_174499)	R 5' – AGCTGTTGCAGTAGCCGAAC – 3'		
ADRA2B	F 5' – GGTCTACCTGCGCATCTACC – 3'	98	89.8
(NM_001206628)	R 5' – GGTGTGGCTGCTTAGACTCC – 3'		
ADRA2C	F 5' – TACTGCAACAGCTCGCTCAAC – 3'	89	89.9
(XM_024993712.1)	R 5' – CTCCGTCGGAAGAGGATGTG – 3'		

4.12 Primer Product Sequencing

Following cloning of amplified primer products into vectors, the vector-integrated AR sequences amplified with primers demonstrating optimal efficiency (see Table 4.1) were sent to the National Research Council (NRC; University of Saskatchewan) for sequencing using fluorescence-based sequencing. The obtained sequences were aligned to the *Bos taurus* AR genes using the NCBI Basic Local Alignment Search Tool (BLAST). Two primers, *ADRA1D*

and *ADRA2C*, amplified products with 99% homology to the expected sequences. ADRA1D had a C → T single nucleotide polymorphism (SNP) at position 90, and ADRA2C had a T deletion at position 79. The remaining primers were all found to have 100% homology to the predicted AR sequences.

4.13 Cell Labeling for Highspeed Sorting

To sort monocytes (CD14+), B Cells (CD21+), Innate Lymphoid Cells (CD335+) and T cells (CD3+), 1x 10^8 PBMCs were re-suspended in 1 mL PBS and 5 μL primary mAb was added (see Table 4.2 for antibody information and concentrations). Cells were incubated for 20 minutes at 4°C, with mixing every 10 minutes. Following labelling with the primary mAb, cells were centrifuged for 8 minutes at 311 X *g* at 4°C, washed twice with 5 mL PBS, and re-suspended in 1 ml PBS. A 5 μL aliquot of R-Phycoerythryin conjugated goat anti-mouse IgG1 polyclonal antibody was added to the cells and cells were incubated in the dark for 20 min at 4°C. The cells were once again pelleted for 8 min at 311 X *g* at 4°C and washed twice with PBS. Following labelling, PBMCs were suspended to a final concentration of 1 x 10^8 cells/mL for high-speed sorting. For sorting PMNs into neutrophils and eosinophils no antibody labeling was required. Bovine eosinophils were identified as auto-fluorescence high in the FL-1 channel, whereas neutrophils were identified as auto-fluorescence low in FL-1 (Anderson et al., Unpublished manuscript). Cytospin slides were prepared with cells sorted based on FL1 autofluorescence using the Cytospin-4 cytocentrifuge (Thermo Shandon, Asheville, NC) and cells were stained with Wright-Giemsa (Hemacolor® Stain Set; Sigma Aldrich).

PMNs could also be gated as distinct subpopulations during flow cytometry when analyzing CD44-PE and CD16-FITC staining, CD44-PE and L-selectin (CD62L)-PerCPCy5.5 staining, and CD44-PE and CD11b-APC staining (Figures 4.5 and 4.6). PMNs were stained with

mAbs using the above method and secondary antibodies used at concentrations shown in Table 4.2. High-speed cell sorting was then performed for each of the PMN subpopulations identified to confirm the subpopulations identified effectively separated neutrophils from eosinophils. Cytospins were prepared for each high-speed sorted PMN subpopulation, stained with Wright-Giemsa, and differential counts performed to determine neutrophil and eosinophil purity.

4.14 Antibody Staining Protocol

Proteins expressed on the surface of PMNs were indirectly labelled using mAbs and mAbs were then detected with fluorochrome-conjugated isotype-specific secondary polyclonal antibodies (Table 4.2). The indirect labelling protocol is briefly described as follows. First, 100 μL of PMNs at a concentration of 1 x 10^7 cells/mL (1 x 10^6 PMNs) were added to 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark). Plates were centrifuged at 349X *g* for 2 minutes at 4°C and the supernatant discarded. In each well, 25 μL each of the four primary mAbs were added at 4X concentrations (see Table 4.2) to each well to a final volume of 100 μL. In isotype control wells, 25 μL matched antibodies for each of the four mAb isotypes were similarly added at matched protein concentrations (see Table 4.2). PMNs were incubated for 20 minutes at 4°C. Following incubation, 100 μL PBS containing 0.03% sodium azide and 0.2% gelatin (PBSG) was added and the cells were centrifuged at 349X*g* for 2 minutes at 4°C. Following discard of the supernatant, cells were washed with 200 μL PBSG. Secondary antibodies were added in the same manner as primary antibodies, incubated for 20 minutes at 4°C and similarly washed.

Following labelling, cells were fixed using 2% formaldehyde and stored at 4°C in the dark until analyzed. Each cell sample was filtered with Falcon 35 μm nylon filter cap tubes (Catalogue # 352235, Corning, New York, NY) prior to analysis.

Table 4.2: Primary and secondary antibodies used to detect PBMC and PMN subsets and PMN activation markers

Antibody Specificity	1º or 2º Antibody	Isotype	Final Concentration	Supplier	Clone Number
CD11b	1°	IgG2b	3.33 µg/mL	VMRD, Inc[1].	MM10A
CD16	1°	IgG2a	1.25 µg/mL	BioRad, Inc.	----------
L-selectin (CD62L)	1°	IgG1	1.25 µg/mL	VMRD, Inc.	BAQ92A
CD44	1°	IgG3	1.25 µg/mL	VMRD, Inc	S-BAG40A
CD3	1°	IgG1	5 µg/mL (sorting) 0.38 µg/mL otherwise	VMRD, Inc	MM1A
CD14	1°	IgG1	5 µg/mL (sorting) 2.55 µg/mL otherwise	VMRD, Inc	MM61A
CD21	1°	IgG1	5 µg/mL (sorting) 2.55 µg/mL otherwise	BioRad, Inc.	MCA1424GA
CD335	1°	IgG1	5 µg/mL		MCA2365GA
Mouse IgG2b Negative Isotype control	1°	IgG2b	3.33 µg/mL	BioRad, Inc.	----------
Mouse IgG2a	1°	IgG2a	1.250 µg/mL	Invitrogen	----------
Mouse IgG1 Isotype Control	1°	IgG1	1.250 µg/mL	Sigma-Aldrich	MOPC 21
Mouse IgG3 Isotype Control	1°	IgG3	1.250 µg/mL	VMRD Inc.	COLIS41B
Goat anti-mouse IgG1-PE	2°	Polyclonal	5 µg/mL	Invitrogen	----------
Goat anti-mouse IgG2b-APC	2°	Polyclonal	0.0625 µg/mL	Southern Biotechnology	----------
Goat anti-mouse IgG2a-FITC	2°	Polyclonal	1:400	Invitrogen	----------
Rat anti-mouse IgG1-PerCP/Cy5.5	2°	Polyclonal	0.3125 µg/mL	BioLegend	----------
Goat anti-mouse IgG3-PE	2°	Polyclonal	0.3125 µg/mL	Southern Biotechnology	----------

1. VMRD Inc. is now the Monoclonal Antibody Center, Washington State University

4.15 Antibodies Used for Flow Cytometry

Each antibody was optimized for specificity and sensitivity by analyzing cell staining intensity (mean fluorescent intensity; MFI) and percentage cells stained over a range of antibody concentrations. Isotype control antibodies were used at a concentration equivalent to the isotype-matched primary mAbs (Table 4.2).

4.16 High-Speed Cell Sorting of PBMC Subsets

Prior to sorting, PBMCs labelled with mAbs were adjusted to a concentration of $1x10^8$ cells/mL and filtered through a 35 μm filter cap tube (BD Falcon, ON, CA) to remove cell aggregates. High-speed sorting was executed with a MoFlo XDP Cell Sorter (Beckman Coulter). The 488 nM Argon laser was used to excite FITC using a 529/28 band-pass filter (FL-1), PE was excited with a 575/25 band-pass filter (FL-2), and PerCP/Cy5.5 excitation was achieved with a 625/26 band-pass filter (FL-3). The 633 nM HeNe laser was used to excite APC using a 670/30 band-pass filter (FL-4). Sample processing and machine setup were as described previously by González-Cano et al. (2014). A primary sort region (R1) displaying FSC and SSC was used to exclude dead cells, contaminating red blood cells, and debris (Figure 4.1A). Cells within R1 were then gated (R2) using FSC height and width to exclude doublets (Figure 4.1B). Cells in R2 were gated based on fluorescence in FL2 (CD14+; R3; Figure 4.1C) to set a positive sort region. Purity of the cells sorted from R3 was then determined by analyzing the percent fluorescent cells in R5 (Figure 4.1D).

Following high-speed cell sorting, selected cell populations cells were centrifuged at 850X g for 8 minutes, the supernatant decanted, and cell pellets were suspended in TRIzol reagent before storing at -80°C until RNA was isolated.

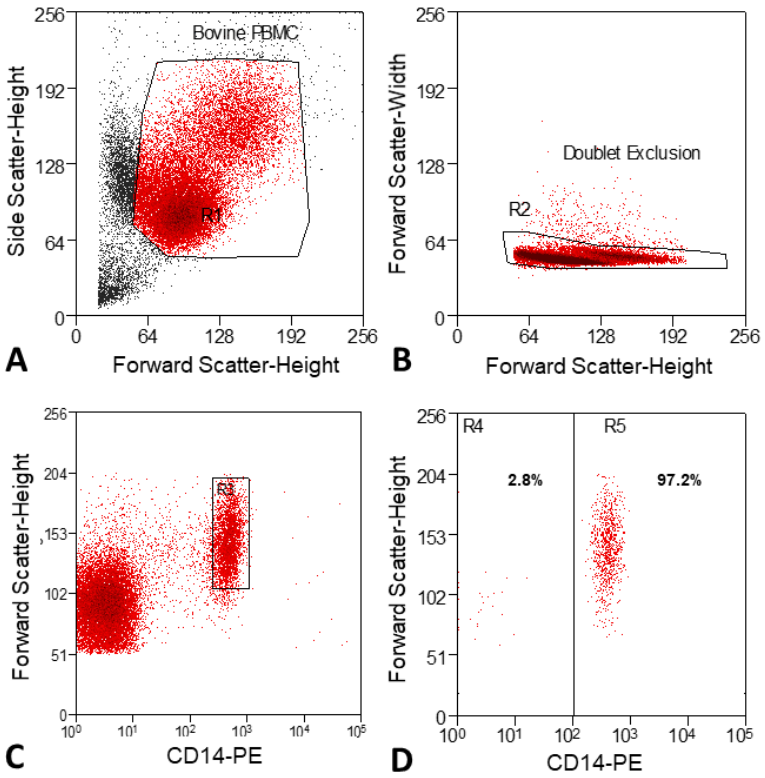


Figure 4.1: High-speed sorting of lineage-specific leukocyte subpopulation from PBMCs. Sorting parameters used for monocytes indirectly labeled with CD14 mAb and R-Phycoerythrin (PE) fluorochrome conjugated goat anti-mouse IgG1 are shown as an example. **A**: PBMCs were first gated (R1) using forward scatter height (size) and side scatter height (complexity) to exclude dead cells and debris. **B**: PBMCs were then gated using FSC height and width (R2) to exclude cell doublets and ensure sorting of single cells. **C**: The sort region for CD14-PE labelled cells (R3) was then set. **D**: Purity of sorted CD14 cells was routinely analyzed and determined to be 97.2%.

4.17 High-Speed Cell Sorting of Neutrophils and Eosinophils

Highly enriched bovine neutrophil and eosinophil populations were obtained using the MoFlo XDP to do a two-way sort of PMNs isolated from bovine blood (initial gating on forward and side scatter; Figure 4.2). The gating strategy for two-way sorting of neutrophils and

eosinophils for RT-qPCR was based on the differential autofluorescence in FL1 for these two cell populations (Figure 4.3). High-speed cell sorting was also used to confirm neutrophils and eosinophils' differentially expressed CD44 and CD16 (Figure 4.5 and 4.6), L-selectin, CD11b (data not shown), and autofluorescence in FL-2 and FL-3 (Figure 4.4) and to validate that flow cytometry analysis regions set using these surface proteins discriminated between neutrophils and eosinophils. Briefly, two-way sort regions for both resting and activated (bovine serum opsonized zymosan [BoZ] + rBoIFNγ) were set using PMNs co-labelled with mAbs specific for various combinations of two surface proteins (Figures 4.5 and 4.6).

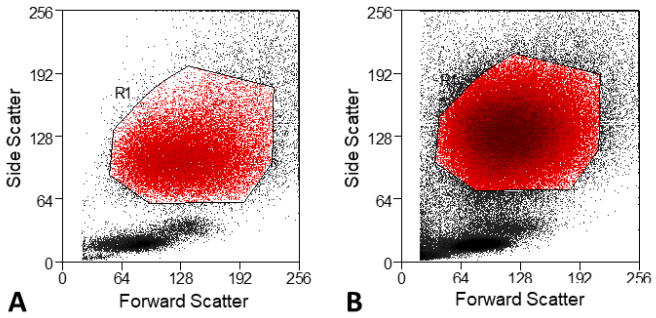


Figure 4.2: Forward scatter (size) and side scatter (complexity) gate (R1) used for sorting resting (A) and BoZ + rBoIFNγ activated (B) PMNs. Region (R1) excluded dead cells and debris. A greater number of events are shown in **B**. A small shift in forward and side scatter was observed when comparing resting and activated PMNs.

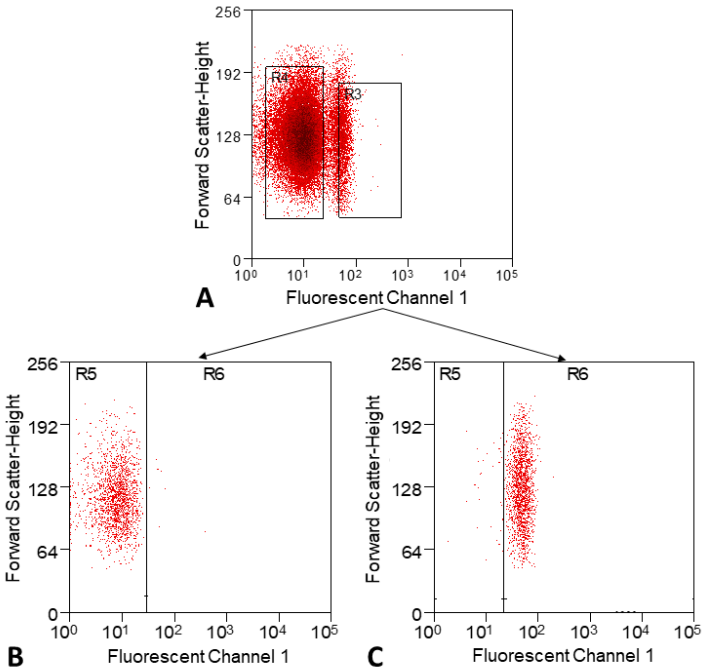


Figure 4.3: High-speed sorting of neutrophils and eosinophils from PMNs based on autofluorescence in channel 1 (FL1). Forward scatter, or cell size, shown on the Y-axis and autofluorescence in FL1 shown on the X-axis. **A:** Two separate sort regions were set using autofluorescence in FL1 and a two-way sort separated FL1 low (**B**) and FL1 high (**C**) cells. Wright-Giemsa staining of cytospins prepared with sorted cells confirmed the FL1 low subpopulation was 99.1% neutrophils and the FL1 high subpopulation was 98.3% eosinophils.

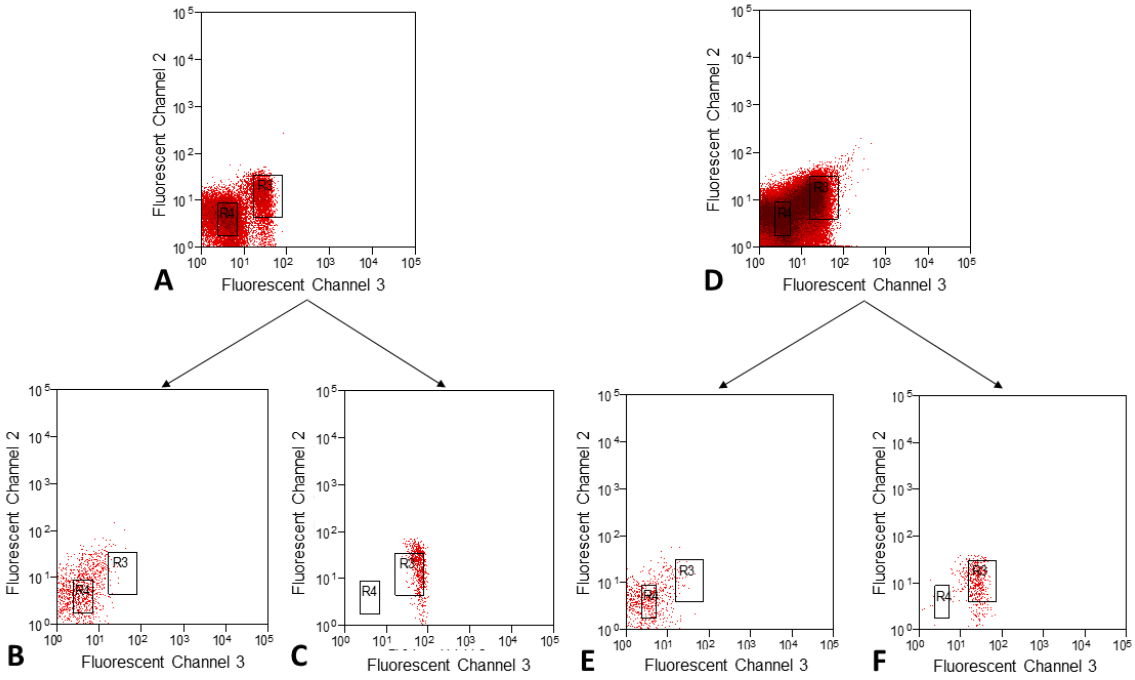


Figure 4.4: High-speed sorting of neutrophils and eosinophils from resting (A, B, C) and BoZ + rBoIFNγ activated (D, E, F) PMNs based on autofluorescence in fluorescent channels 2 and 3. A: Resting PMNs were gated for two separate sort regions using FL2 and FL3 autofluorescence and a two-way sort separated FL2 and FL3 low (**B**) and FL2 and FL3 high (**C**) cells. **D:** BoZ + rBoIFNγ activated PMNs were gated into two sort regions using FL2 and FL3 autofluorescence and a two-way sort separated FL2 and FL3 low (**E**) and FL2 and FL3 high (**F**) cells. Wright-Giemsa staining of cytospins confirmed sort purity for resting neutrophils was 100% and resting eosinophils was 98.0%. Sort purity of BoZ + rBoIFNγ activated neutrophils was 92.7%, and BoZ + rBoIFNγ activated eosinophils was 84.7%.

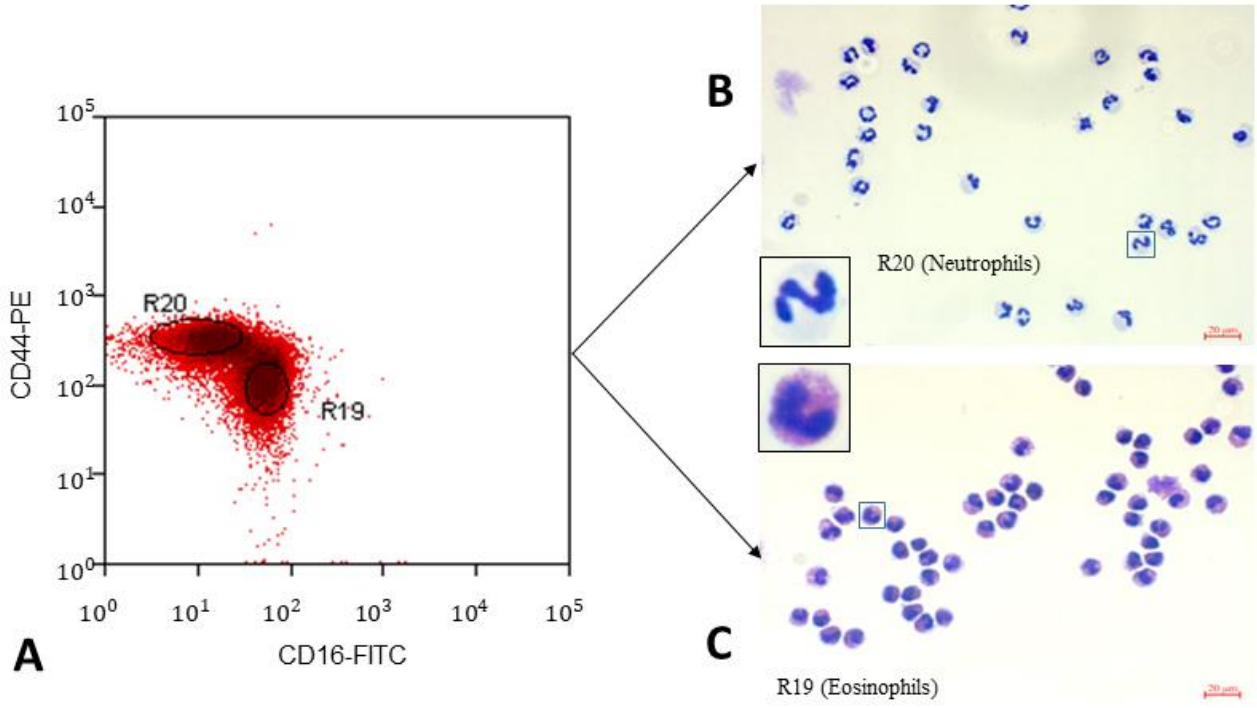


Figure 4.5: High-speed sorting of PMNs based on CD16 and CD44 expression. A: Two sort regions were set using CD16- FITC (FL1) and CD44- PE (FL2) and a two-way sort separated CD16 low, CD44 high (**B**) and CD16 high, CD44 low (**C**) cells. **B**: Wright-Giemsa stained CD44 high, CD16 low cells confirming this population was 98.6% neutrophils (see inset for segmented nucleus and clear cytoplasm). **C**: Wright-Giemsa stained CD44 low, CD16 high cells confirming this population was 98.4% eosinophils (see inset for characteristic pink cytoplasmic granules). Scale bar = 20 μm; Magnification = 400X.

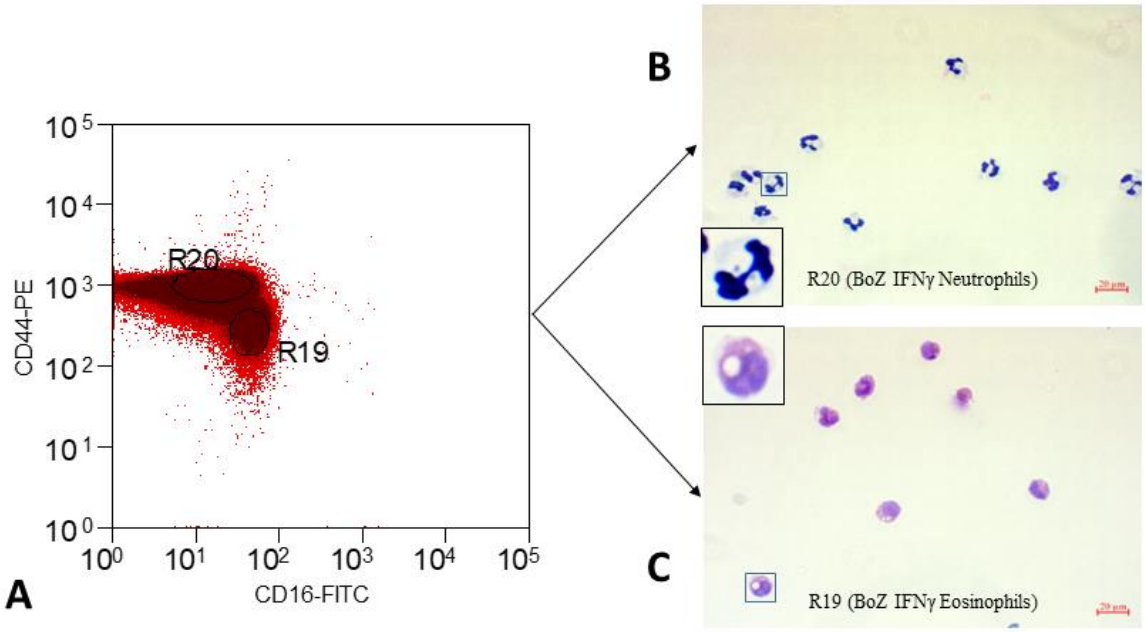


Figure 4.6: High-speed sorting of BoZ + rBoIFNγ activated PMNs based on CD16 and CD44 expression. A: Two sort regions were set using CD16- FITC and CD44- PE and a two-way sort separated CD16 low, CD44 high (**B**) and CD16 high, CD44 low (**C**) cells. **B**: Wright-Giemsa stained CD44 high, CD16 low cells confirmed 98.1 % were neutrophils (Inset: BoZ + rBoIFNγ activated neutrophils contain vacuoles containing bovine serum opsonized zymosan). **C**: Wright-Giemsa stained CD44 low, CD16 high cells confirmed 94.9 % were eosinophils with characteristic pink granules (Inset). Vacuoles were observed in BoZ rBoIFNγ activated eosinophils consistent with the uptake of BoZ particles. Scale bar = 20 μm; Magnification = 400X.

4.18 Stress Trial for Analysis of AR Gene Expression in Blood Leukocytes

The study included four groups (n = 10/group) of 6-7 month old, suckling, female Hereford-cross calves (Goodale Farm, University of Saskatchewan). Group A remained in the pasture with their dams and had *ad libitum* access to brome-alfalfa hay and water. This group were controls for possible stress responses while calves were restrained for blood collection (Controls). Group B

calves also remained with their dams but were moved from pasture to a dry lot with *ad libitum* access to brome-alfalfa hay and water. This group served as controls for changing the environment from pasture to a dry lot (Controls). Group C calves were separated from their dams following sampling on Day 0 and housed in a dry lot adjacent to Group B (Weaning). Group D calves were separated from their mothers following sampling on Day 0, transported in a livestock trailer for approximately 5 h (Weaning + Transportation) and then co-housed with Group C calves. Blood was collected from calves in all groups on Day 0, and Days 2, 4, 8, 14 and 28 post-weaning. Serum and blood leukocytes were isolated and aliquots of 10 X 10^6 blood leukocytes were pelleted and snap-frozen in liquid nitrogen before storing at -80 ^{o}C. Cell pellets were used for RNA extraction and RT-qPCR analysis of AR gene expression.

4.19 Zymosan Opsonization

Zymosan A from *Saccharomyces cerevisiae* (Sigma Aldrich) was opsonized with bovine serum using the method described by Roth and Kaeberle (1981) with minor adaptations. Briefly, fresh bovine serum was prepared by collecting blood in 10 ml SST Vacutainers (Becton Dickinson) and blood was incubated at room temperature for 1 hour. Blood was then centrifuged at 2000 X *g* for 15 minutes to separate serum. One gram Zymosan A was suspended in 100 mL cold Hank's Buffered Saline Solution (HBSS) supplemented with Ca^{++} and Mg^{++}. The zymosan suspension was vortexed at maximum speed for 1 minute and 100 mL of 10 mg/mL Zymosan A was added to 100 mL fresh bovine serum. The mixture was stirred vigorously for 1 hour at room temperature using a stir bar and magnetic stirrer and then centrifuged at room temperature for 10 minutes at 250 X *g*. The supernatant was discarded before suspending the pellet in 200 mL 0.01M EDTA and stirring for 15 minutes at room temperature to prevent conglutination. The mixture was then centrifuged at room temperature for 10 minutes at 250 X *g* and the supernatant

discarded. The pellet was re-suspended in 200 mL 0.01M EDTA and stirred for 15 minutes a room temperature. The mixture was again centrifuged at room temperature for 10 minutes at 250 X *g* and the supernatant discarded. The pellet was re-suspended in 100 mL HBSS with Ca^{++} and Mg^{++} to a final concentration of 10 mg/mL and 500-1000 μL aliquots of bovine serum opsonized zymosan (BoZ) were stored at -20°C. Immediately prior to use, aliquots were thawed and vortexed at high speed for 2 minutes.

4.2 PMN Culture Media

PMNs were cultured using media previously optimized for cell viability during a 48 to 72 h culture period (Whale et al. 2006). Briefly, phenol-red free AIM-V Medium (Thermo Fisher Scientific) was supplemented with 20% fetal bovine serum (FBS; SAFC Biosciences Inc., Cat #12103C-500mL, Lot #14G420) and 50 μM β-2 mercaptoethanol (Sigma Aldrich).

4.21 Treatment of PMNs with Adrenergic Agonists

Freshly isolated PMNs were plated in 6-well plates at a final concentration of 1 million cells /mL for a total of 5 million cells/well in 5 mLs culture media. Adrenergic agonists were then added at final concentrations varying between 0-100 nM (freshly diluted from powder to prevent oxidation and degradation) and cells were incubated for 30 minutes at 39°C. For resting PMNs, the cells were incubated an additional hour at 39°C before collecting cells for analyses (Figure 4.7A). For activated PMNs, following the 30-minute pre-incubation with adrenergic agonists, 10 ng/mL recombinant bovine IFNγ (rBoIFNγ) and 6.25 μg/mL BoZ were added and the cells were incubated for another hour at 39°C before collecting cells for analyses (Figure 4.7B). Adrenergic agonists (all full agonists) tested included (±) epinephrine hydrochloride (Sigma Aldrich), DL-norepinephrine hydrochloride (Sigma Aldrich), (R)-(−)-Phenylephrine

hydrochloride (Sigma Aldrich) for α1-ARs, Dexmedetomidine hydrochloride (Sigma Aldrich) for α2-ARs, and (-)-Isoproterenol hydrochloride (Sigma Aldrich) for β-ARs.

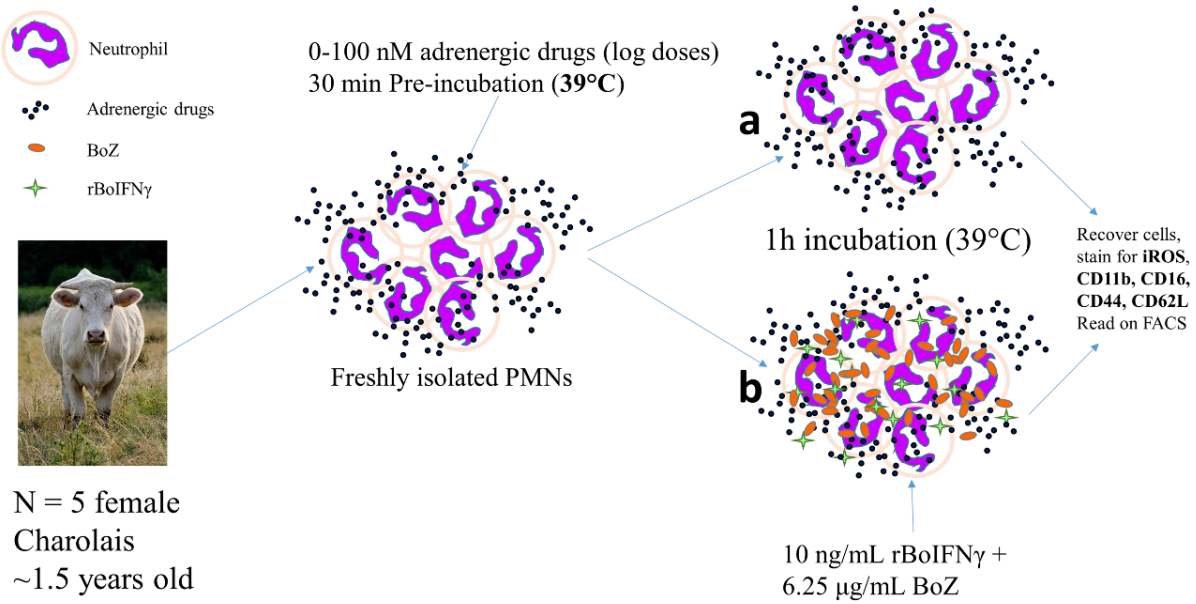


Figure 4.7: Protocol used to evaluate the effect of adrenergic agonists on bovine neutrophils and eosinophils. Each adrenergic agonist was titrated using 10-fold dilutions (100, 10, 1, 0.1 and 0 nM) in the physiological range for E and NE in cattle. The adrenergic compounds used were all considered full agonists. Cultured PMNs were incubated for 30 minutes at 39°C with individual adrenergic agonist, and **A.** resting PMNs were then cultured for another hour before being collected for analysis of neutrophil and eosinophil activation. **B.** For PMN activation, 10 ng/ml rBoIFNγ and 6.25 µg/mL BoZ were added during the last hour of incubation. With this activation protocol, an absence of cell aggregation was confirmed on cytospin slides, cell recovery from tissue culture plates was determined to be >85%, and cell viability was determined to be >99% as determined by propidium iodide (PI) staining. Following recovery

from cultures, PMNs were stained for iROS and stained with mAbs to quantify surface expression of CD11b, CD16, CD44 and L-selectin.

4.22 Recovery of Cultured PMNs

Briefly, culture media was aspirated and gently discharged into the well 4-5 times to re-suspend non-adherent cells before collecting all media from each well in the 6-well culture plate (Corning Life Sciences, Inc. Kennebunk, ME). Following media collection, 3 mL of 39°C 0.1% EDTA prepared in PBS (pH 7.3) was added to each well and plates were incubated for 5 minutes at 39°C in a humidified atmosphere with 5% CO_2. The EDTA solution was collected and pooled with the previously collected media. Another 3 ml of 39°C 0.1% EDTA was added to each well. Plates were incubated at 39°C for 10 minutes before collecting the EDTA solution and pooling with previously collected media. Cells were pelleted by centrifuging at 300 X *g* for 8 minutes at 4°C. Supernatant was discarded and the cell pellet re-suspended in 3 mL PBS containing 0.03% sodium azide and 0.2% gelatin (PBSG) adjusted to a pH of 7.3. Cells were pelleted again by centrifuging at 300 X *g* for 8 minutes at 4°C before discarding the supernatant and re-suspending cells in 400 μL PBS (PBS; pH of 7.3).

4.23 Detection of Intracellular Reactive Oxygen Species

The 2',7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Inc., Eugene, OR) dye was used to quantify intracellular reactive oxygen species (iROS) activity in PMNs. Following removal of the acetate group by iROS and intracellular esterases, the dye fluoresces and is detectable by flow cytometry (Invitrogen Reactive Oxygen Species Detection Reagents Protocol, 2006). DCFDA was dissolved in DMSO (Sigma Aldrich) at a concentration of 20mM and stored in 20 μL aliquots at -20°C. A fresh aliquot was thawed and diluted to 1 μM in PBS

immediately prior to use. DMSO concentrations in assays were tested to confirm they did not reduce cell viability. DCFDA dye concentration and incubation period with cells was optimized for bovine PMNs. The optimized protocol for bovine PMNs is as follows: one million bovine PMNs were added in 100 µl culture medium to each well of a 96-well plate. Cells were pelleted by centrifuging at 349 X *g* for 2 minutes at room temperature and supernatant was discarded. Cells were re-suspended in 100 µL PBS containing 1 µM DCFDA and incubated for 4 minutes at 39°C. Cells were pelleted by centrifuging at 349 X *g* for 2 minutes at room temperature and re-suspended in 200 µL 37°C neutrophil culture medium and incubated for 2 minutes at 39°C to allow hydrolyzation of acetate groups. Cells were pelleted, supernatant discarded, and cells re-suspended in 200 µL PBS. FL-1 fluorescence of cells was analyzed with a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) with 10,000 events acquired for each sample.

4.24 Detection of PMN Apoptosis and Necrosis

PMN apoptosis and necrosis was analyzed using flow cytometry to quantify staining by anti-Annexin V antibodies and propidium iodide (PI) using the APC Annexin V Apoptosis Detection Kit with PI (Biolegend, Inc., San Diego, CA). Cell staining was performed according to kit instructions with the exception that PI was added immediately prior to analyzing each sample with the flow cytometer.

4.25 Epinephrine and Norepinephrine: Dose Ranges and Pre-Incubation

For experiments with E and NE, a dose range of 0.1 nM to 100 nM was chosen with 10-fold incremental dilutions. The range of E and NE doses selected was based on information regarding basal and stress associated levels of E and NE in humans, rats, and cattle (Table 4.3; Adapted from Buhler et al. 1978).

Table 4.3: Basal and stressed levels of E and NE

	Basal (nM)	**Stress** (nM)
Human Epinephrine	0.306	----
Bovine Epinephrine	0.349	----
Rat Epinephrine	0.955	16.4 to 133.6
Human Norepinephrine	0.898	----
Bovine Norepinephrine	1.200	----
Rat Norepinephrine	3.009	17.7 to 94.1

4.26 Flow Cytometry Analysis of Neutrophil and Eosinophil Subpopulations

Flow cytometric analysis of cultured PMNs and data acquisition was performed using a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ). A primary analysis region was set using FSC and SSC (R1) to exclude dead cells, contaminating red blood cells, and debris (not shown) and 10,000 gated events were acquired. The 488 nM laser was used to excite FITC, PE and PerCP/Cy5.5. FITC fluorescence was acquired in FL-1 with a 530/30 filter. PE fluorescence was acquired in FL-2 with a 585/42 filter, and PerCP/Cy5.5 fluorescence in FL-3 with a 670 LP filter. The 635 nM laser was used to excite APC and fluorescence was detected in FL-4 with a 661/16 filter. Manual compensation was used to eliminate fluorescence overlap between channels. Data was analyzed using the BD CellQuest Pro program (version 6.0).

Fluorescence from DCFDA was detected in FL-1 and neutrophil and eosinophil subpopulations in PMNs were gated separately based upon differential auto-fluorescence in either FL-2 and FL-3 (see Figure 4.5 and Figure 4.6). Regions for selecting positive CD16, CD44, L-selectin and CD11b fluorescence were set using isotype-matched negative control mAbs (see Table 4.2). Neutrophils and eosinophils were discriminated by gating on CD44-PE and CD16-FITC (see Figure 4.8 and 4.9), as these regions were confirmed by high-speed cell

sorting to provide the highest purity for each PMN subpopulation. Example are shown for separate gating of neutrophils (Figure 4.8) and eosinophils (Figure 4.9) based on CD44-PE and CD16-FITC.

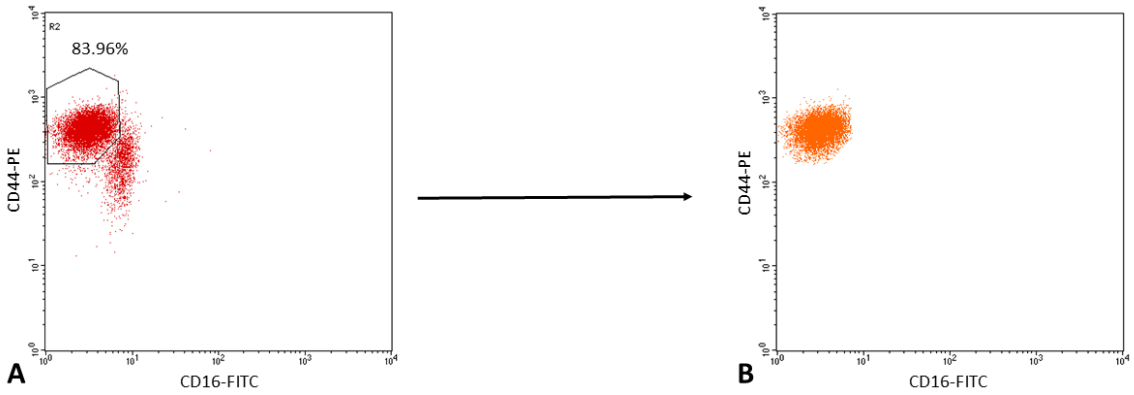


Figure 4.8: Dot scatter plots of unstimulated PMNs gated for CD16-FITC low and CD44-PE high cells. Cells in this region (R2) were confirmed to be neutrophils (**B**) and this region was used to analyze neutrophil responses to adrenergic agonists.

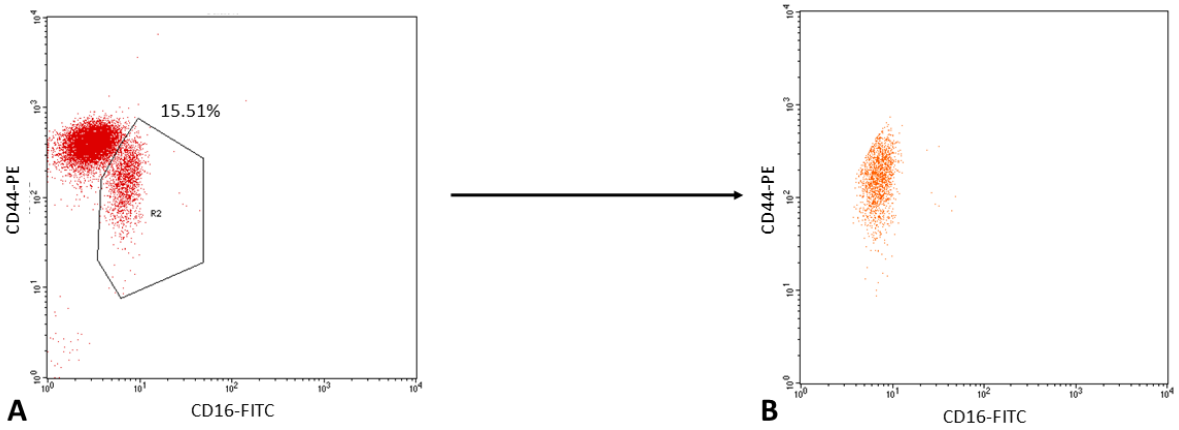


Figure 4.9: Dot scatter plots of unstimulated PMNs gated for CD16- FITC high and CD44-PE low cells. Cells in this region (R2) were confirmed to be eosinophils (**B**) and this region was used to analyze eosinophil responses to adrenergic agonists.

4.27 Analysis of PMN Morphology to Identify Neutrophils and Eosinophils

PMNs and high-speed sorted subpopulations were diluted to 2 x 10^5 cells/mL in PBS supplemented with 1% bovine serum albumin (BSA). A 100 µl aliquot of cells was deposited onto glass slides (Fisher Scientific) using the Cytospin-4 cytocentrifuge (Thermo Shandon, Kalamazoo, MI) set at 28 X *g* for 5 minutes. Slides were air dried before staining with the Harleco® Hemacolor Stain Set (Merck; Darmstadt, Germany). When slides were dry, a coverslip was mounted over the cytospin using Cytoseal 60 (Richard–Allan Scientific, Kalamazoo, MI) as a mounting medium. Differential counts of neutrophils and eosinophils were performed using an Olympus CX31 (Olympus Corporation, Tokyo, Japan) light microscope and digital images of cells were captured using the Zeiss Axio Observer 7 microscope (Zeiss, Oberkochen, Germany).

4.28 Statistical Analysis

Adrenergic receptor gene expression data for leukocytes isolated from whole blood was analyzed using a one-way analysis of variance (ANOVA). Adrenergic receptor gene expression data for PBMC, PMNs, neutrophils and eosinophils were analyzed using a matching two-way ANOVA with gene and cell type as variables. When significant differences were detected then post-hoc analysis testing for differences in expression of individual genes and among cell types was completed using Tukey's multiple comparison test. Data was similarly analyzed for the expression of AR genes over time in blood leukocytes using a two-way, repeated measures ANOVA with time and treatment as variables.

Activation of bovine PMNs with 10 ng/mL rBoIFNγ and 6.25 µg/mL BoZ was performed at three different time points for each animal, and PMN iROS, CD11b, CD16, CD44, and L-selectin responses for each of the 5 animals were averaged across time points and used to compare the effect of different treatments. Changes in PMN activation responses for cells

isolated from individual animals were calculated as a percentage relative to unstimulated cells and comparisons between neutrophil and eosinophil responses were analyzed using a student's t test. Comparisons between neutrophil and eosinophil expression of basal levels of activation markers were performed similarly.

Data for treatment of PMNs with adrenergic receptor agonists were analyzed using a matching two-way ANOVA with dose and treatment as variables. Dunnett's multiple comparisons test was used to analyze dose-dependent effects of treatment, whereas Sidak's multiple comparisons test was used to compare responses among multiple agonists. All analyses were performed using Graphpad Prism 8.3.1 statistical software (Graphpad Software, San Diego, CA).

5.0 RESULTS

5.1 ADRENERGIC RECEPTOR GENE EXPRESSION IN BOVINE LEUKOCYTES

5.1.1 Adrenergic Receptor Transcript Expression in Leukocytes Isolated from Whole Blood

My first objective was to determine which AR genes were expressed at a detectable level in bovine blood leukocytes and whether transcript abundance varied significantly when comparing among individual AR genes. The RT-qPCR analysis of AR gene expression was performed using RNA extracted from leukocytes isolated from whole blood. A detectable level of transcript ($Cq < 35$) was present for all 9 ARs but significant ($p < 0.05$) variation in transcript abundance was observed when comparing among the 9 genes (Figure 5.1). The α2A-AR gene was unique in that its transcript abundance was significantly ($p < 0.01$) greater than all other AR genes. The α1A-AR was expressed at the next highest level with transcript abundance significantly ($p < 0.05$) greater than all other α1-, α2-, and β-ARs. A lower but similar transcript abundance was observed for the α1B-, α2C, and β2-AR genes. Finally, transcript abundance was detectable but lowest for α1D-, α2B-, and the β1- and β3-AR genes.

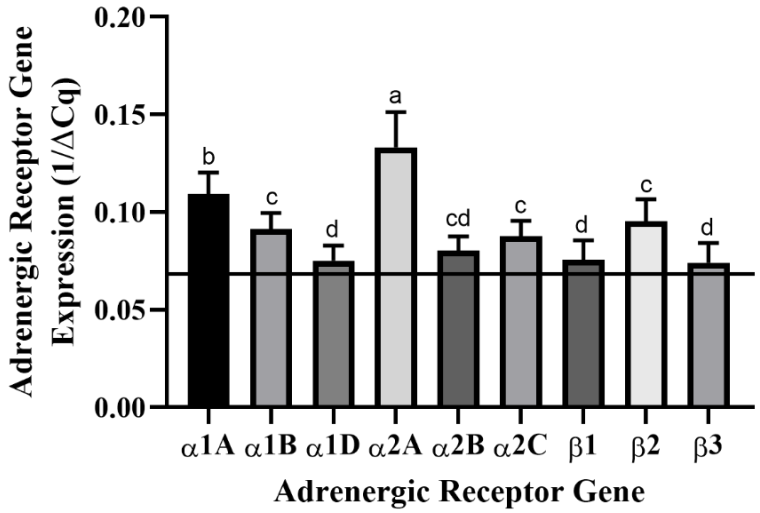


Figure 5.1: Expression of nine known bovine AR genes in leukocytes isolated from whole blood. Gene expression data is expressed as 1/ΔCq and presented as the mean + 1 SD (n = 31 cattle). The horizontal line indicates the lower cut-off for detection of transcript (>35 PCR cycles). A one-way ANOVA was used to compare transcript abundance among individual AR genes. Genes that are significantly ($p < 0.05$) different in transcript abundance are identified by different letters (a, b, c, d).

5.1.2 Effect of Stress on Adrenergic Receptor Expression in Bovine Leukocytes

Addressing my second objective analyzing AR gene expression in bovine leukocytes, I investigated whether stress associated with maternal separation (weaning) and transportation significantly altered AR gene expression in blood leukocytes. For this study, RNA was isolated from whole blood leukocytes following red blood cell lysis. The RT-qPCR analyses again confirmed transcript was detectable for all 9 ARs in blood leukocytes and there were no significant differences in the expression of the 9 AR genes when comparing calves suckling dams on pasture (Group A) versus suckling calves moved with their dams into a corral (Group B) (data not shown). This indicated a change in environment associated with moving animals

from pasture to a dry lot did not significantly alter AR gene expression. Furthermore, there were no significant temporal changes in the expression of AR genes within either group of suckling calves throughout the one-month sampling period. Therefore, data from the two groups of suckling calves (n = 20) were combined for all subsequent analyses.

Expression of 4 of the 9 AR genes changed significantly ($p < 0.05$) in blood leukocytes isolated from calves following maternal separation, either with (Group D) or without (Group C) transportation. A significant ($p < 0.01$) increase in β2-AR gene expression was observed on Days 2 and 4 post-weaning in Group C (weaned), and on Day 2 post-weaning in Group D (weaned + transport) relative to the control group (Figure 5.4). A significant ($p < 0.05$) increase in α2A-AR gene expression was also observed in Group C (weaned) on Day 4 and 8 post-weaning and on Day 8 post-weaning in Group D (weaned + transport) when compared to the control group (Figure 5.3). On Day 28 post-weaning significant ($p < 0.01$) increases in the expression of both β1- and β3-AR (Figure 5.4) were observed in Group D (weaned+transport) relative to the controls. For the β3-AR, there was also a significant difference on Day 28 in Group D (weaned+transport) relative to Group C (weaned), providing evidence the two stressors differentially regulated AR gene expression. Thus, maternal separation (Group C) was associated with significant changes in AR gene expression by blood leukocytes during the first 8 days post-weaning but combining maternal separation with transportation was associated with both short-term and long-term effects on blood leukocyte AR gene expression that were apparent 4 weeks post-weaning.

Significant changes in AR gene expression in blood leukocytes following the stress of maternal separation and transportation could result from either altered AR gene transcription or changes in the cellular composition of the blood leukocyte population (Hickey, Drennan and

Earley, 2003; Yagi *et al*., 2004; Ishizaki and Kariya, 2010; O'Loughlin *et al*., 2012). Therefore, I investigated whether individual blood leukocyte lineages differed significantly in AR gene expression.

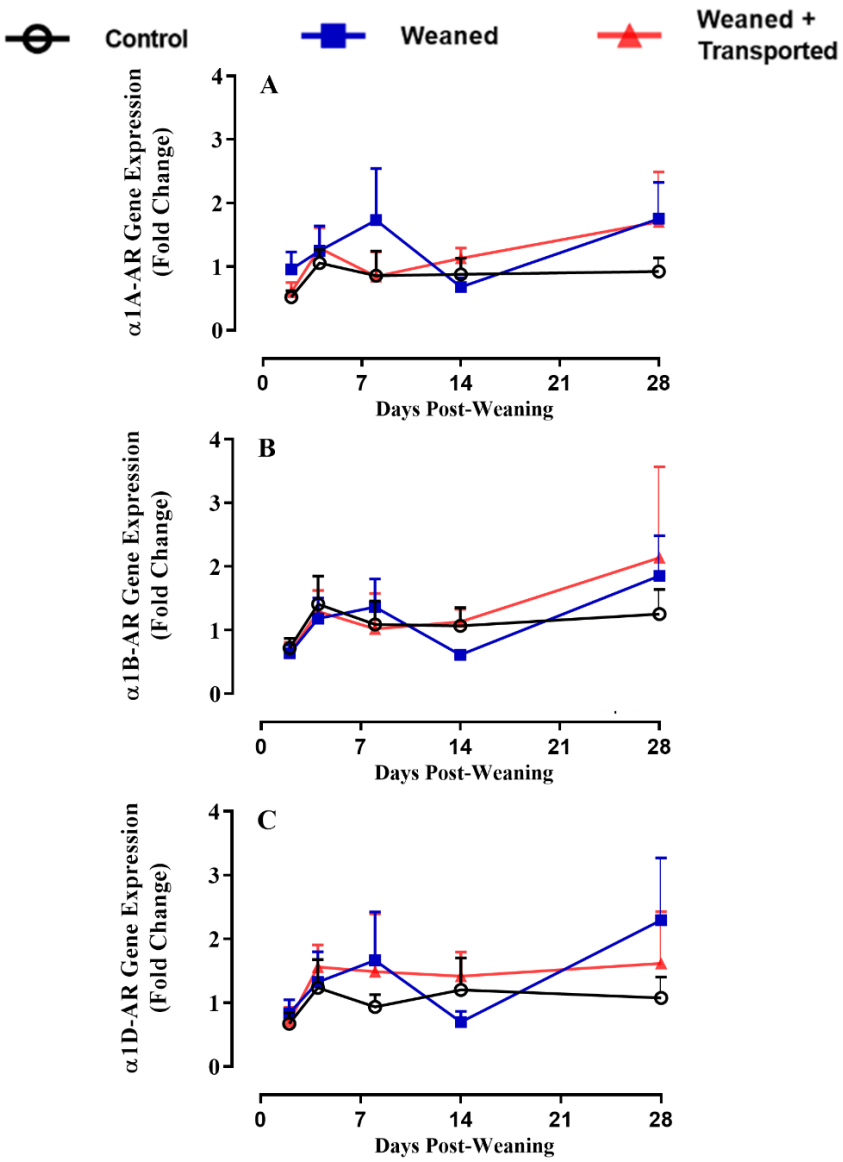


Figure 5.2: Temporal changes in α1-AR gene expression in blood leukocytes following weaning, with or without transportation. Gene expression was quantified with RT-qPCR and data for each animal was expressed as fold change relative to Day 0 (prior to weaning). Gene expression was analyzed for three α1-AR (**A, B, C**) genes. A one-way ANOVA was used to

compare the three treatment groups at each time point and significant differences between the two treatment groups and the control group are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

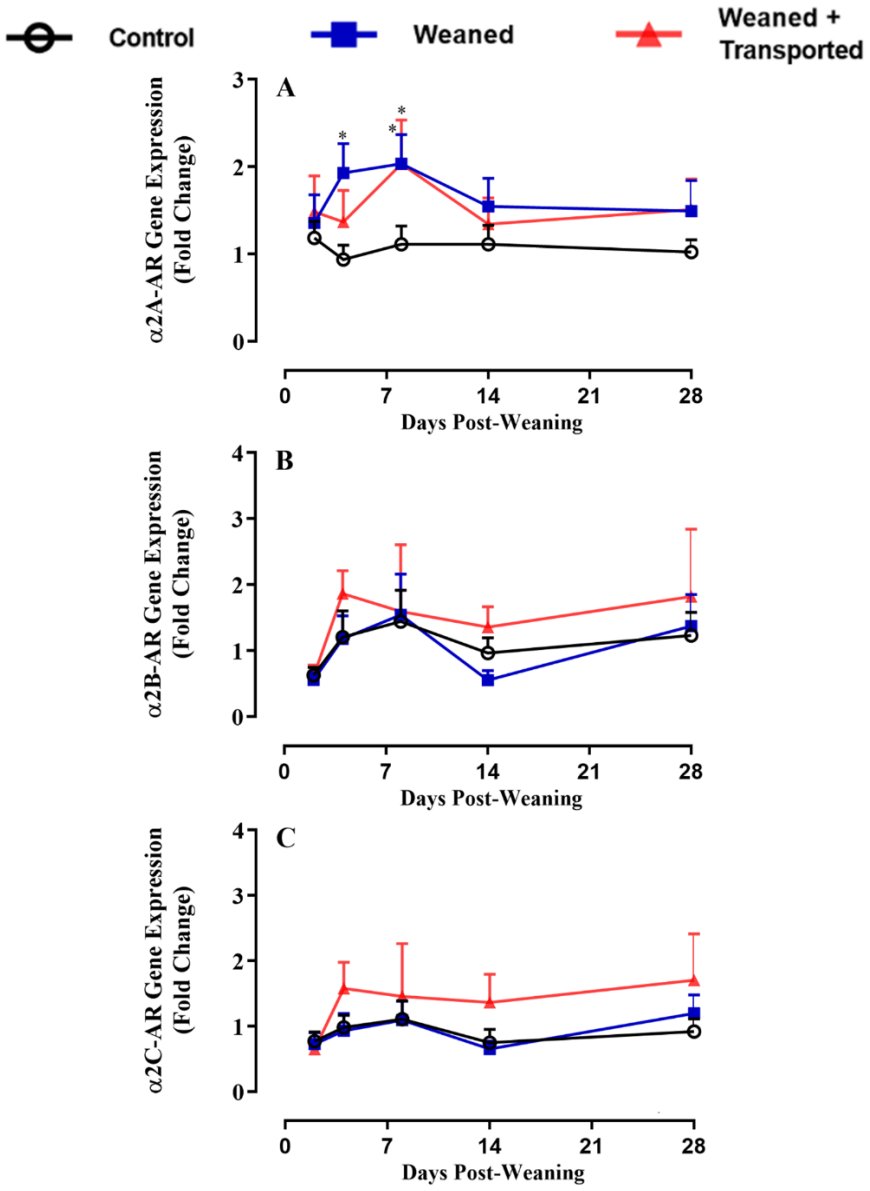


Figure 5.3: Temporal changes in α2-AR gene expression in blood leukocytes following weaning, with or without transportation. Gene expression was quantified with RT-qPCR and data for each animal was expressed as fold change relative to Day 0 (prior to weaning). Gene expression was analyzed for three α2-AR (**A, B, C**) genes. A one-way ANOVA was used to

compare the three treatment groups at each time point and significant differences between the two treatment groups and the control group are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

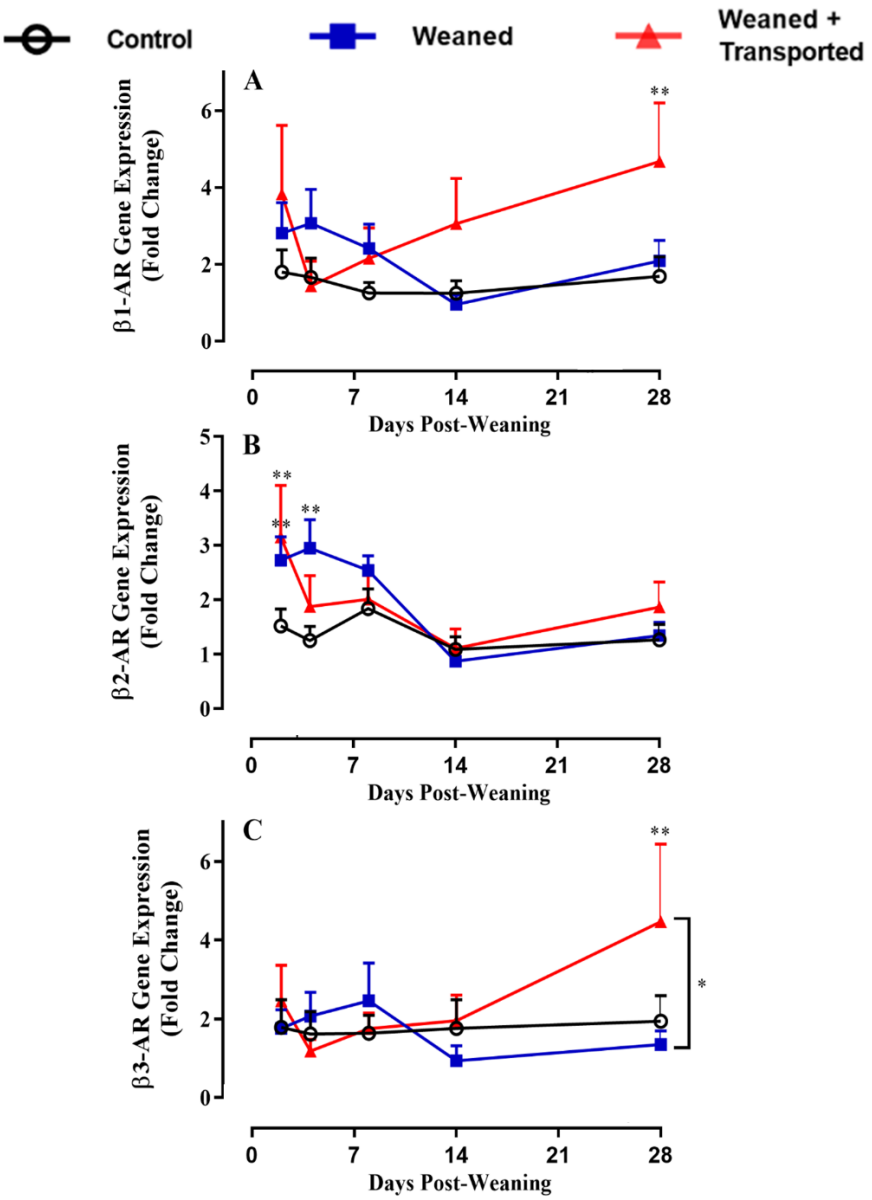


Figure 5.4: Temporal changes in β-AR gene expression in blood leukocytes following weaning, with or without transportation. Gene expression was quantified with RT-qPCR and data for each animal was expressed as fold change relative to Day 0 (prior to weaning). Gene expression was analyzed for three β-AR (**A, B, C**) genes. A one-way ANOVA was used to

compare the three treatment groups at each time point and significant differences between the two treatment groups and the control group are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

5.1.3 Adrenergic Receptor Transcript Expression in Leukocyte Subpopulations

5.1.3.1 Adrenergic Receptor Transcript Expression in PMNs and PBMCs

Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear (PMNs) leukocytes are the two major leukocyte lineages that can easily be separated when studying blood leukocytes. When comparing PBMCs and PMNs, the RT-qPCR analysis revealed similar transcript abundance for all AR genes except for the α2A-AR gene (Figure 5.5). The α2A-AR gene was expressed at a significantly ($p < 0.01$) higher level in PBMCs than PMNs. Furthermore, within PBMCs the α2A-AR gene was expressed at a significantly ($p < 0.05$) higher level than all other AR genes and expression of the α1A- and β2-AR genes was similar but significantly ($p < 0.05$) higher than the α1B-, α1D-, α2B-, α2C-, β1- and β3-AR genes. Within PMNs, the α1A-, α2A-, and β2-AR genes were expressed at similar but significantly higher levels than all other AR genes. The α1B-, β1- and β3-AR genes were expressed at a similar but lower level that was significantly ($p < 0.05$) greater than the α1D-AR gene.

Collectively, this data confirmed all 9 AR genes were expressed at detectable levels in both PBMCs and PMNs (Figure 5.5). Furthermore, there was a significant difference in AR gene expression when comparing PBMCs and PMNs.

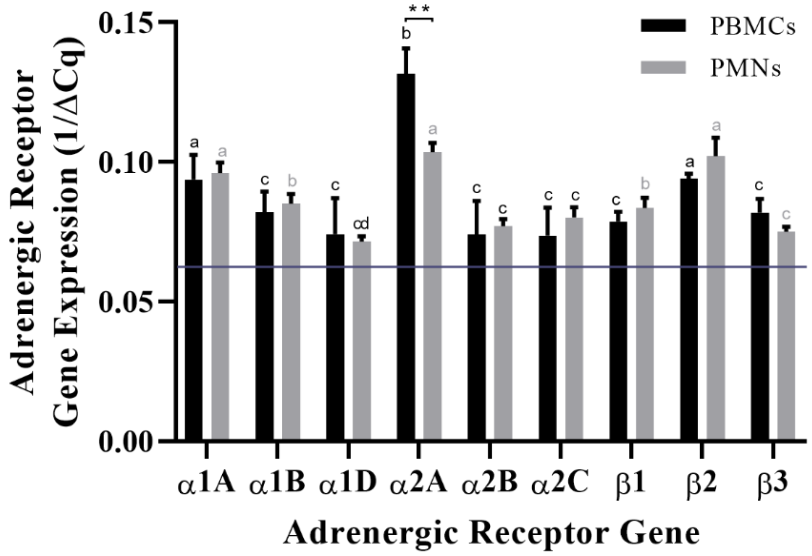


Figure 5.5: Expression of nine known bovine AR genes in PBMCs and PMNs. Gene expression is expressed as 1/ΔCq and presented as the mean + 1 SD (n = 5 cattle). The horizontal line indicates the lower cut-off for detection of transcript (>35 PCR cycles). A two-way ANOVA was used to compare AR transcript abundance within and between the two leukocyte subpopulations. Genes that are significantly ($p < 0.05$) different in transcript abundance within PBMCs and within PMNs are identified by different letters (a, b, c, d). Significant differences in the expression of individual AR genes when comparing between PBMCs and PMNs are indicated** ($p < 0.01$).

5.1.3.2 Adrenergic Receptor Gene Expression in PBMC Subpopulations

The variation in AR gene expression observed within PBMCs suggested that even greater variation in AR gene expression may be apparent if PBMCs were separated into distinct

lymphocyte and myeloid cell lineages. Thus, high-speed cell sorting was used to isolate highly enriched (> 95% purity) T cell ($CD3^+$), B cell ($CD21^+$), and innate lymphoid cell (ILC; $CD335^+$) populations and monocytes ($CD14^+$ cells). RNA was extracted from each purified subpopulation to analyze AR gene expression.

As expected, much greater differences in AR gene expression were apparent when comparing within and among the various PBMC subpopulations (Figure 5.6). These differences were most pronounced for several AR genes, including the α2A- and β2-AR genes. Expression of the α2A-AR gene was significantly ($p < 0.05$) higher in B cells than all other lymphocyte subpopulations and monocytes. Expression of the α2A-AR gene was also significantly ($p < 0.01$) higher in ILCs relative to monocytes, which had the lowest α2A-AR transcript abundance. Significantly ($p < 0.01$) higher β2-AR transcript expression was detected in ILCs relative to other lymphocyte subpopulations and monocytes. Monocytes, however, expressed the β2-AR gene at a significantly ($p < 0.05$) higher level than either B cells or T cells. Monocytes and B cells did not express detectable levels of transcript for either the α1D-, α2B- or α2C-AR genes. Otherwise, all AR genes were detectable in the PBMC subpopulations.

Within monocytes, the β2-AR gene was the most highly expressed ($p < 0.05$), followed by the α2A-AR gene ($p < 0.05$), and the α1B- and β1-AR genes ($p < 0.05$). Expression of the α1A-AR gene was similar to the later three genes mentioned. The α1B- and β1-AR genes were expressed at a significantly ($p < 0.05$) higher level than the α1D- and α2B-AR genes, which were not detectable. Expression of the α2C- and β3-AR genes were also close to the limit of detection.

Within B cells, the α2A-AR gene was expressed at a higher level ($p < 0.05$) than all the other AR genes. The β1-, β2-, α1A-, and α1B-AR genes were similarly expressed at the next

highest level, followed by β3-AR at a significantly ($p < 0.05$) lower level. Expressed near the limit of detection were the α2B-, α2C- and α1D-AR genes.

$CD335^+$ ILCs, which include conventional natural killer or NK cells and non-conventional T cells, expressed the β2-AR gene at a significantly ($p < 0.01$) higher level than all other AR genes. The α1A and α2A-AR genes displayed similar levels of expression at a significantly ($p < 0.05$) lower level than the β2-AR gene but significantly ($p < 0.05$) higher than the α1B-AR gene. Low but detectable levels of transcript were present for the α2B-, α2C-, β1-, and β3-AR genes.

Purified T cells displayed the least variation in AR gene expression. The AR gene expressed at the highest level was α2A but the β2- and α1A-AR genes were also expressed at similar levels. The α1B-, β1-, and α2C-AR are expressed at a significantly ($p <0.05$) different level from the α2A-AR. Expression of the α1D-, α2B-, and β3-AR genes was barely detectable.

Collectively, these data reveal that while monocytes, B cells, T cells, and ILCs are often analyzed together as PBMCs, there is marked variation in AR gene expression within and among these subpopulations. Differential expression of the α2A- and β2-AR genes was most marked when comparing among PBMC subpopulations, suggesting different capacities for cells within each subpopulation to respond to adrenergic agonists and that a change in the frequency of cells within an individual subpopulation could alter transcript abundance when analyzing AR gene expression in blood leukocytes.

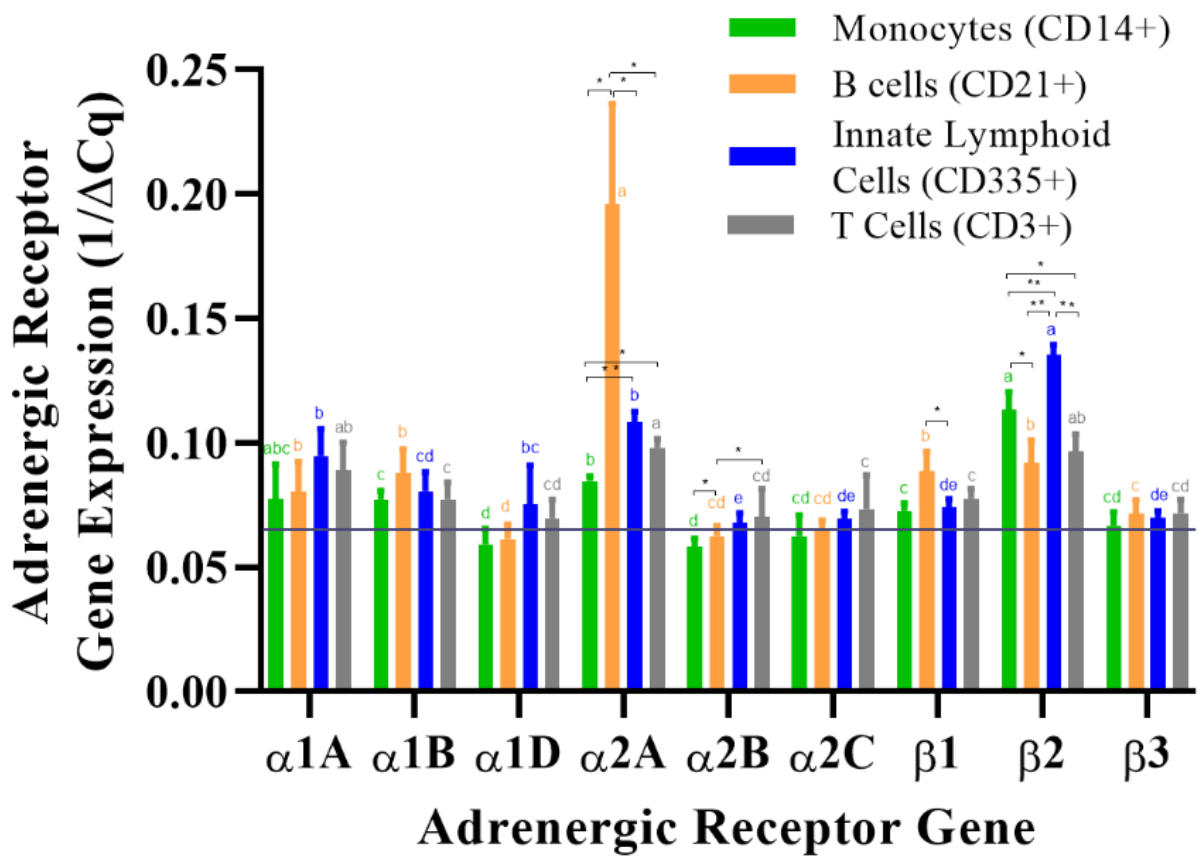


Figure 5.6: Expression of the nine known bovine AR genes in lymphocyte subpopulations and monocytes. Lymphocyte subpopulations analyzed included T cells ($CD3^+$), B cells ($CD21^+$), and innate lymphoid cells ($CD335^+$) and monocytes were identified as $CD14^+$ cells. Data expressed as 1/ΔCq and presented as mean + 1 SD (n = 5 cattle). The horizontal line indicates the cutoff used to define detectable gene expression (>35 PCR cycles). A 2-way ANOVA was used to compare AR gene expression within and among the purified subpopulations. Genes that are significantly ($p < 0.05$) different in transcript abundance within individual PBMC subpopulations are identified by different letters (a, b, c, d). Significant differences in the expression of an individual AR gene when comparing among PBMC subpopulations are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

5.1.3.3 Adrenergic Receptor Gene Expression in PMN Subpopulations

Marked differences in AR gene expression observed among PBMC subpopulations suggested significant differences in AR gene expression may also occur when comparing PMN subpopulations. PMNs in bovine blood are primarily neutrophils but eosinophils comprise an average of 3.6% of blood leukocytes in healthy animals, varying between 0-10.8% (George, Snipes and Lane, 2010). Differences in neutrophil and eosinophil autofluorescence in FL-1 were validated as a parameter that could be used with high-speed cell sorting to generate highly purified (> 95% purity) neutrophil and eosinophil subpopulations (Andersen et al. unpublished data). As a result, discrimination between neutrophils and eosinophils based on FL-1 fluorescence was used to sort these PMN subpopulations. Validation for the effective separation of neutrophils and eosinophils based on FL-2 and FL-3 autofluorescence is demonstrated in the Materials and Methods (Figure 4.4). However, significantly ($p < 0.01$) higher eosinophil autofluorescence is observed in all channels on the FACSCalibur (Figure 5.7). Greater autofluorescence by eosinophils is most pronounced in FL-3.

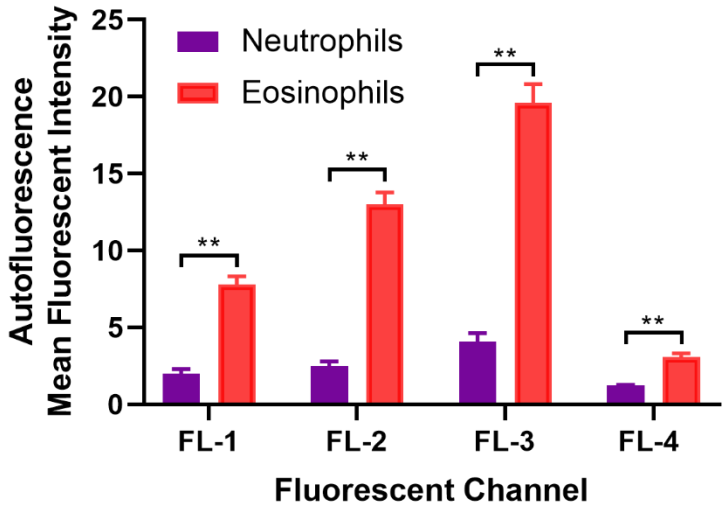


Figure 5.7: Autofluorescence of bovine neutrophils and eosinophils in four fluorescence (FL) channels. Autofluorescence is expressed as mean fluorescent intensity of each PMN subpopulation and presented as the mean + 1 SD (n = 5 cattle). Multiple t-tests were used to compare mean fluorescent intensity between the two PMN subpopulations. Significant differences in neutrophils and eosinophils autofluorescence are indicated ** ($p < 0.01$).

RNA extracted from high-speed sorted neutrophils and eosinophils was used to complete RT-qPCR analysis of AR gene expression (Figure 5.8). Neutrophils expressed a detectable level of transcript for all 9 ARs with few significant differences when comparing among individual AR genes. Transcript was most abundant for the α2C-AR gene but was not significantly different from α2A-, α2B-, or the β2- and β3-AR genes. Expression of the α2C-AR gene was, however, significantly ($p < 0.05$) greater than all the α1-AR genes and the β1-AR gene. In contrast, eosinophils displayed a more varied expression when comparing among AR genes, with expression of the α1B- and α1D-AR genes at the limit of detection. In contrast, the α2A- and β2-AR genes were expressed at a similar but significantly ($p < 0.01$) higher levels than all other AR genes. The remaining AR genes were expressed at similar but lower levels except for β1-AR, which was expressed at a significantly ($p < 0.05$) lower level than the α1A-AR gene.

A comparison of neutrophils and eosinophils revealed significant ($p < 0.01$) differences in the expression of all AR genes except for the α1A-AR gene (Figure 5.8). Neutrophils displayed significantly ($p < 0.01$) greater expression of the α1B- and α1D-AR genes, the α2B- and α2C-AR genes, and the β1- and β3-AR genes when compared to eosinophils. In contrast, eosinophils expressed the α2A- and β2-AR genes at significantly ($p < 0.01$) higher levels than neutrophils. Collectively, these analyses (n = 6) revealed that neutrophils and eosinophils,

although often grouped together as PMNs, display multiple differences in AR gene expression that may impact their responses to adrenergic hormones or agonists. These data strengthen the argument that neutrophils and eosinophils should be studied as individual cell populations and literature reporting the responses of PMNs to adrenergic agonists should not be directly extrapolated to neutrophils.

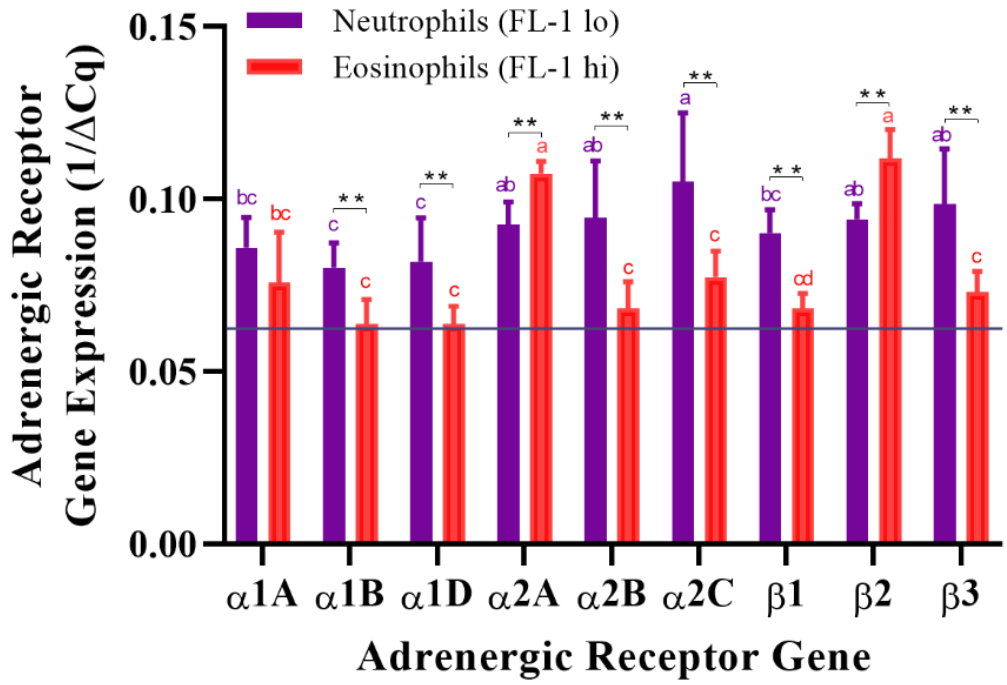


Figure 5.8: Expression of AR genes in purified bovine neutrophils and eosinophils. Gene expression data is expressed as 1/ΔCq and presented as the mean + 1 SD (n = 6 cattle). The horizontal line indicates the lower cut-off for detection of transcript (>35 PCR cycles). A 2-way ANOVA was used to compare AR transcript abundance expression both within and between the two PMN subpopulations. Genes that are significantly ($p < 0.05$) different in transcript abundance within neutrophils and within eosinophils are identified by different letters (a, b, c, d). Significant differences in the expression of individual AR genes when comparing neutrophils and eosinophils are indicated as ** ($p < 0.01$).

In conclusion, numerous significant differences in transcript abundance of individual AR genes were observed when comparing among bovine leukocyte subpopulations (Figures 5.5, 5.6, and 5.8). Of note, the α2A-AR gene was the most highly expressed AR gene in B cells, ILCs, and eosinophils. The β2-AR gene was also highly expressed in ILCs, monocytes, and eosinophils. Among all the leukocyte subpopulations analyzed, neutrophils had the highest expression of the α2B-, α2C-, and β3-AR genes. These many differences in AR gene expression raise the possibility that individual leukocyte populations may respond very differently during a stress response characterized by increased release of epinephrine and norepinephrine.

5.2 BOVINE NEUTROPHILS AND EOSINOPHILS

5.2.1 Optimization of PMN Culture and Activation Conditions

The analysis of AR gene expression in bovine leukocytes was followed by a more focused analysis of bovine PMNs to address the question which ARs regulate the function of both resting and activated PMNs. To address this question, bovine PMN culture, activation, and recovery conditions were optimized for flow cytometric analyses of single cells. Assays to measure PMN activation were also established, and culture conditions optimized for the analysis of PMN activation, viability, and recovery. The media used for all assays was AIM-V media supplemented with 20% FBS and β-2 mercaptoethanol (Whale et al. 2006) (see Materials and Methods) and PMNs were activated by co-stimulation with a combination of BoZ and rBoIFNγ for 1 hour at 39°C (normal bovine body temperature). Furthermore, the expression of L-selectin, CD44, CD16, CD11b, and iROS were assessed as quantitative measures of PMN activation (Figure 5.15 and 5.16).

The initial evaluation of BoZ as a method for PMN activation included assessing viable cell recoveries following BoZ treatment. Maximizing the number of viable PMNs recovered was considered important to ensure a representative cell population was analyzed. Both short-term (2h) and long-term (18h) PMN activation were analyzed. Increasing the dose of BoZ resulted in a numerical decrease in viable PMN recovery and this difference was significant ($p < 0.05$) when comparing media alone to 0.1 mg/mL BoZ (Figure 5.9). No significant dose-dependent differences in viable cell recovery were observed when PMNs were cultured for 18 h, even though there was again a numerical decline in the recovery of viable cells as the dose of BoZ increased (Figure 5.9). Furthermore, there were no significant differences in viable PMNs recovery at 2h versus 18h when comparing individual doses of BoZ (Figure 5.9).

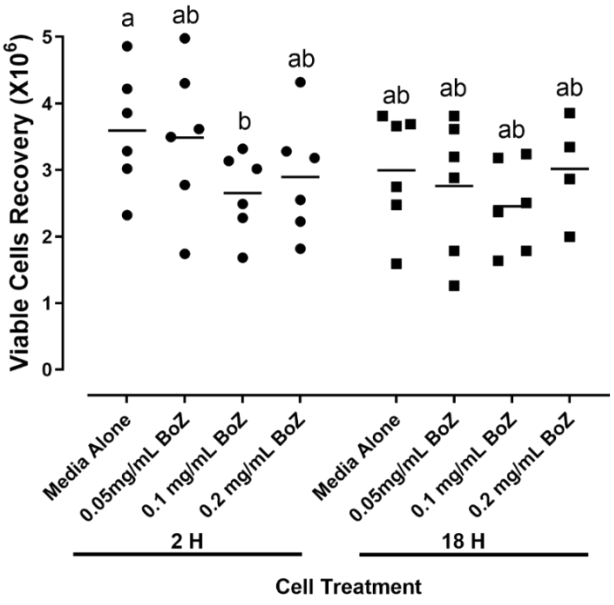


Figure 5.9: Recovery of viable PMNs following activation with BoZ. PMNs (5×10^6 cells/well) were stimulated with different doses of opsonized zymosan (BoZ) for either 2 or 18 h prior to recovery and analysis of viable cell number. Data presented are values from independent experiments (n = 5) and the horizontal bar represents the mean value. Viable PMN recovery was

calculated by multiplying total cell counts with a hemocytometer by the proportion of cells negative for Annexin-V and Propidium Iodide staining. A two-way ANOVA was used to compare viable cell recovery for different BoZ doses within and between the two incubation periods. There were no significant differences when comparing between 2 and 18 h but significant ($p < 0.05$) differences in viable cell recovery at two hours are indicated (a, b).

Evaluating BoZ as a method for activating PMNs included measuring the dose-dependent induction of iROS production. A dose-dependent increase in iROS production was observed as BoZ was increased from 0.01 mg/ml to a maximum of 0.2 mg/ml, although there was a marginal increase in fluorescence when comparing 0.1 versus 0.2 mg BoZ/mL (Figure 5.10). This analysis confirmed BoZ increased iROS production in PMNs.

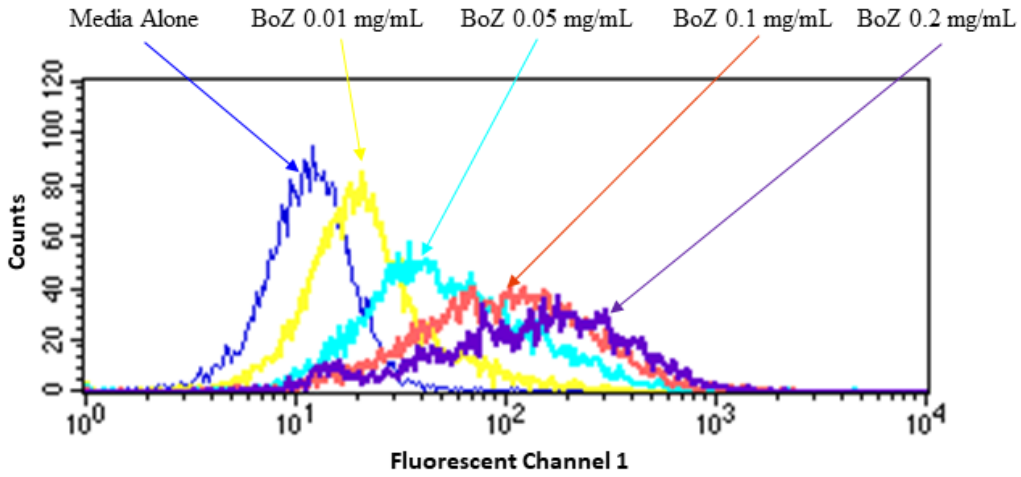
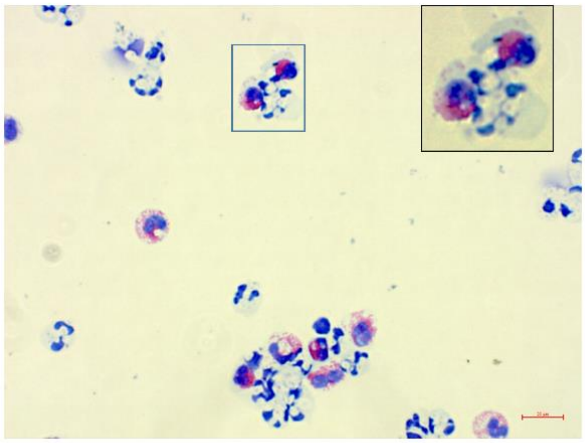


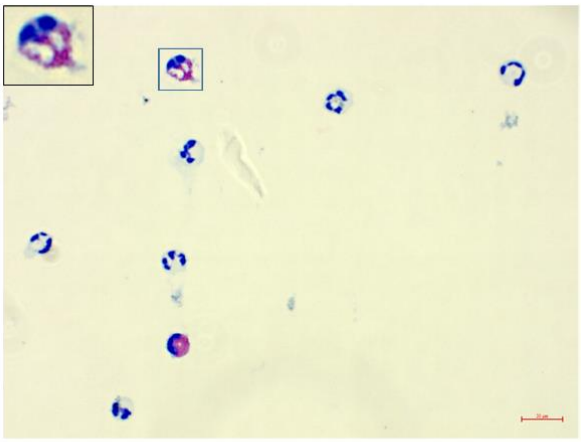
Figure 5.10: Intracellular (i)ROS production following PMN incubation with BoZ. Following a 2 h incubation in medium alone or with various doses of BoZ (0.01 mg/ml to 0.2 mg/ml) cells were stained with propidium iodide (PI) to exclude dead (PI+) cells and DCFDA to measure iROS (FL1). Data presented are histograms of DCFDA fluorescence measured with flow cytometry.

Although BoZ had a dose-dependent effect on iROS production, as the dose of BoZ was increased there was also increased cell clumping (as demonstrated on cytospin slides; Figure 5.11) and cell death (as demonstrated on FACS profiles; data not shown). Cells were filtered through a Falcon 35 μm filter to reduce cell clumping and PI staining was used to exclude dead cells from analysis. However, it was critical the recovered PMNs remained a single cell suspension to facilitate effective flow cytometric analysis of changes in the expression of cell surface markers and iROS. The generation of doublets or larger cell clumps increases the fluorescent signal for each event acquired by the flow cytometer. A method of cell activation which activated bovine PMNs without increasing cell aggregation or cell death was required.

The final protocol for activating PMNs used was a combination of BoZ and recombinant bovine interferon gamma (rBoIFNγ). The two substances have a synergistic effect on bovine PMN activation (Sample and Czuprynski, 1990). Minimal cell clumping was observed following stimulation with rBoIFNγ and 6.25 ug/ml BoZ when cells were examined on cytospin slides (Figure 5.11); viable cell recovery from one-hour cultures was >85%, and cell viability was >99% as determined by propidium iodide (PI) staining (data not shown).



A



B

Figure 5.11: PMNs activated with (A) 0.05 mg/mL of BoZ vs. B) 6.25 µg/mL of BoZ and 10 ng/mL of rBoIFNγ. A: PMNs were incubated for two hours at 37°C with 0.05 mg/mL of BoZ (see inset for greater consumption of BoZ alongside cell clumping). **B:** PMNs were incubated for one hour at 39°C with 6.25 µg/mL of BoZ and 10 ng/mL of rBoIFNγ (see inset for less consumption of BoZ and no cell clumping). Scale bar = 20 µm; Inset magnification = 400X.

As observed in Figure 5.12, a substantial increase in both forward scatter (size) and side scatter (complexity) is observed in bovine PMNs activated for 2 h with 0.05 mg/mL of BoZ. Part of this may be a result of neutrophil and eosinophil phagocytosis of BoZ, which was confirmed by microscopy (Figure 5.9), but a proportion of this increase may also be the result of cell clumping.

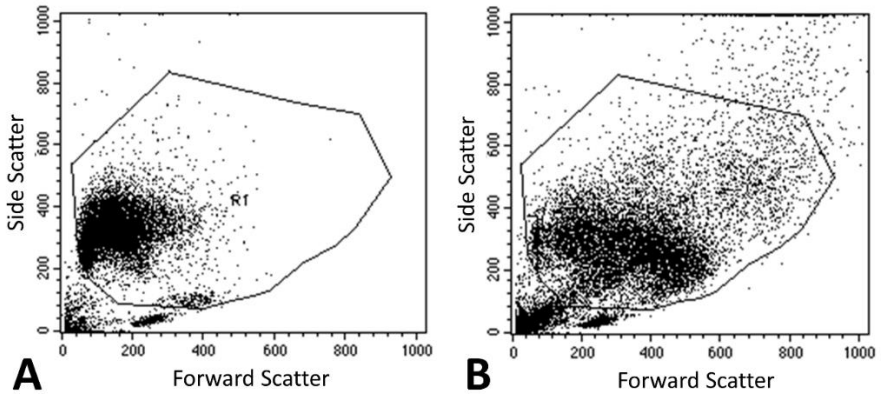


Figure 5.12: Dot scatter plots of cell size (Forward Scatter) and complexity (Side Scatter) of resting (A) and BoZ activated (B) PMNs. Resting PMNs were incubated in medium alone for 2 hours at 37°C and activated PMNs were incubated for two hours with 0.05 mg/mL of BoZ. R1 excludes dead cells and debris.

A smaller increase in forward scatter (size) and side scatter (complexity) was observed when PMNs were activated with the combination of BoZ and IFNγ (Figure 5.12) when compared to the much larger dose of BoZ alone (Figure 5.13).

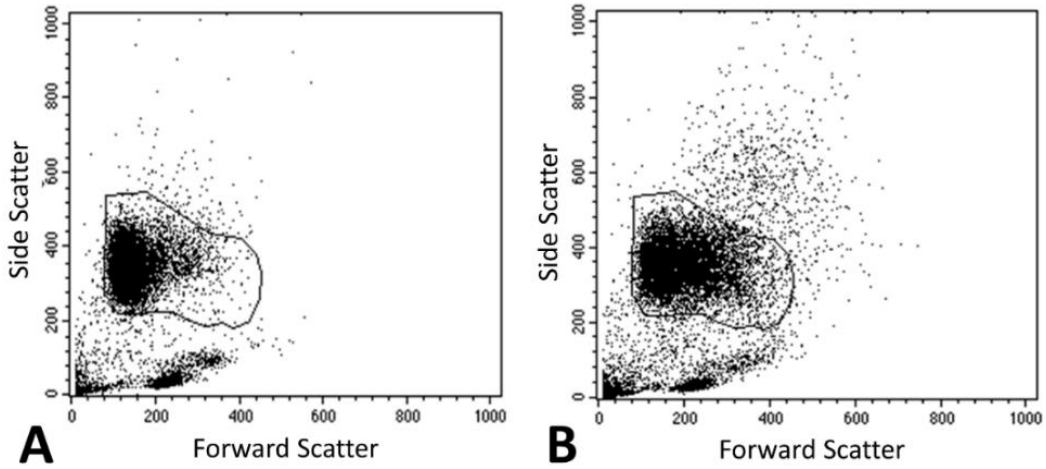


Figure 5.13: Dot scatter plots of cell size (Forward Scatter) and complexity (Side Scatter) of resting (A) and BoZ rBoIFNγ activated (B) PMNs. Resting PMNs were incubated in AIM-V medium for one hour at 39°C. PMNs were activated by incubation for one hour with 10 ng/mL rBoIFNγ and 6.25 μg/mL of BoZ. R1 excludes dead cells and debris.

The final procedure for PMN activation (Figure 4.7) was optimized to maximize recovery of a viable single cell suspension.

5.2.2 Percentage Neutrophils and Eosinophils in PMN Preparations

Five calves were sampled at three different times (March to June, August to September, and late September) and gating on autofluorescence was used to identify eosinophils and neutrophils within the PMN preparation. The proportion of eosinophils and neutrophils in PMN preparations was observed to vary with the time of the year (Figure 5.14). Significantly ($p < 0.01$) fewer eosinophils were observed when comparing the period of March to June (spring) to

the August to early September and late September (autumn) intervals. The percentage eosinophils in PMNs varied from a low of 1.8% to a high of 29.4%. Similarly, the percentage neutrophils in PMNs varied from a high of 97.8% to as low as 70.28%. Given the common practice of assuming eosinophils contribute little to PMN responses, these data indicate that in Saskatchewan, at certain times of the year, eosinophils may have a substantial influence when the collective response of PMNs is being measured.

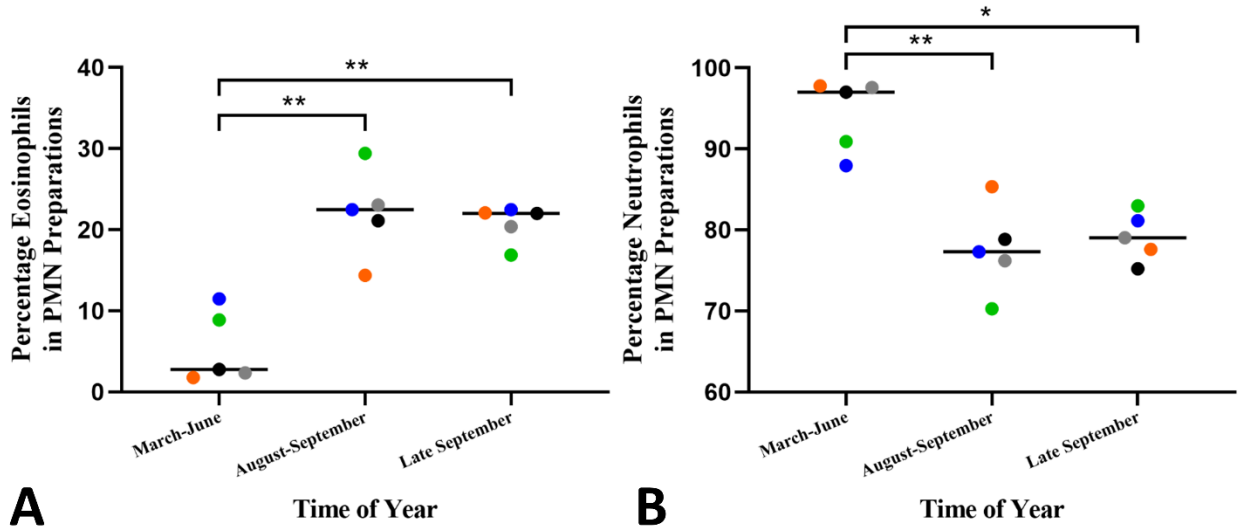


Figure 5.14: Percent (A) eosinophils and (B) neutrophils in PMNs isolated from the same five animals at different times of the year. Data presented are values for individual animals and the horizontal bar represents the mean. Individual animals are identified by different colours. A one-way ANOVA was used to compare the percent eosinophils and neutrophils at different times of the year. Eosinophils were identified by using flow cytometry to gate on autofluorescence high cells in fluorescent channel 2 and 3. Neutrophils were identified by using flow cytometry to gate on autofluorescence low cells in fluorescent channel 2 and 3. Significant differences are indicated ** ($p < 0.01$).

5.2.3 Neutrophil and Eosinophil Basal Expression of Activation Markers

Neutrophils and eosinophils were both > 98% positive for iROS, CD11b, L-selectin and CD44 (data not shown). The mean fluorescent intensity of iROS and CD11b was similar for both resting bovine neutrophils and eosinophils (Figure 5.15). L-selectin expression, however, was significantly ($p < 0.01$) greater on eosinophils than neutrophils and CD44 was expressed at a significantly ($p < 0.01$) higher level on neutrophils than eosinophils. This difference in CD44 expression was subsequently used to discriminate between these two PMN subpopulations.

CD16 expression was detectable on few resting eosinophils (< 1.0%) and was also expressed at a low but detectable level on a minor number of neutrophils (< 30.0%) (data not shown). However, higher eosinophil autofluorescence in FL-1 provided another effective parameter to discriminate between eosinophils and neutrophils.

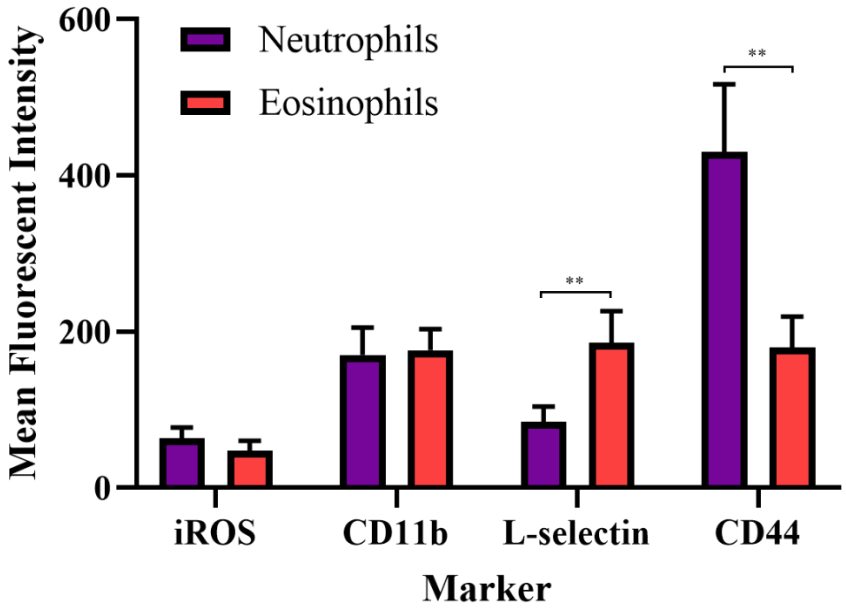


Figure 5.15: Expression of activation markers in bovine neutrophils and eosinophils following culture for one hour at 39°C. Data presented are mean fluorescence intensity + 1 SD

(n = 5 cattle). Seasonal variation in iROS expression of neutrophils and CD44 expression of eosinophils may contribute to the inter-animal variation that was observed. Multiple t-tests were used to compare expression of individual markers on neutrophils and eosinophils. Significant differences between neutrophils and eosinophils are indicated as ** ($p < 0.01$).

5.2.4 Neutrophil and Eosinophil Activation Responses

Treatment with BoZ + rBoIFNγ for one hour significantly alters the expression of multiple activation markers on neutrophils (Figure 5.16; Table 5.1 and 5.2). These changes include significantly ($p < 0.01$) increased iROS, increased CD11b, decreased L-selectin, and decreased CD44 expression. This activation protocol also significantly ($p < 0.01$) alters eosinophils, with increased ROS, increased CD11b, and decreased L-selectin. No change in CD16 expression was observed for either activated neutrophils or eosinophils. These changes in activation markers for both neutrophils and eosinophils confirmed both PMN subpopulations were activated when co-stimulated with BoZ + rBoIFNγ (Table 5.1 and 5.2).

Significant differences in neutrophil and eosinophil responses to BoZ + rBoIFNγ were also observed (Figure 5.16; Table 5.1 and 5.2). CD11b increased significantly ($p < 0.01$) more on activated eosinophils than neutrophils. L-selectin decreased significantly ($p < 0.01$) more on activated eosinophils than neutrophils and CD44 decreased significantly ($p< 0.01$) on neutrophils but not eosinophils. Thus, for two of the activation markers eosinophils had a more pronounced reaction to BoZ + rBoIFNγ than neutrophils. As the percentage eosinophils increases within a PMN population these cells may make a substantial contribution when measuring the collective response of BoZ + rBoIFNγ activated PMNs.

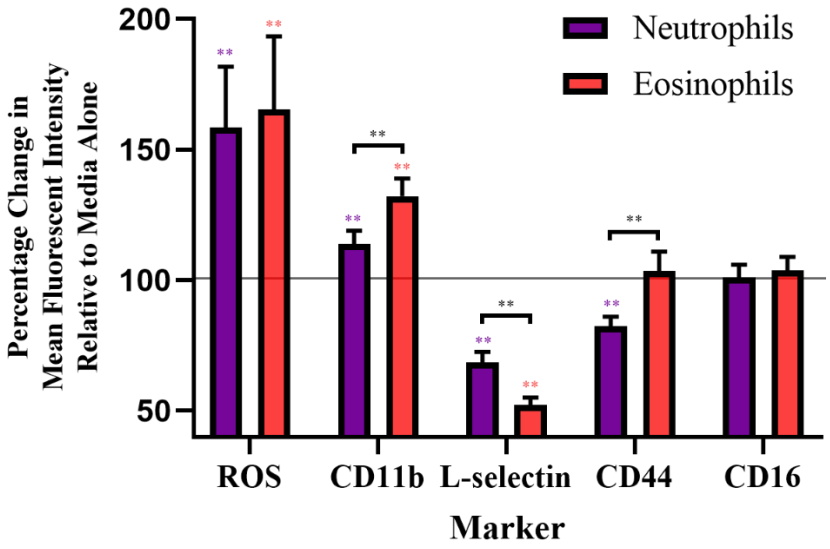


Figure 5.16: Expression of activation markers on bovine neutrophils and eosinophils following incubation with 10 ng/mL rBoIFNγ and 6.25 µg/mL BoZ for one hour. Data presented are mean fluorescence intensity + 1 SD (n = 5 cattle) and are expressed as a percent of baseline values for neutrophils and eosinophils cultured in media alone. A student's t-test was used to compare expression levels of individual markers on activated neutrophils and eosinophils. Significant differences are indicated as ** ($p < 0.01$).

Table 5.1: Bovine neutrophil and eosinophil ROS, CD11b, CD16, L-selectin, and CD44 values following co-stimulation with rBoIFNγ and BoZ.

PMN Activation Marker	Neutrophil Change In Expression[1]	Neutrophil p-value	Consistent with Activation[2]	Eosinophil Change In Expression[1]	Eosinophil p-value	Consistent with Activation
iROS	+ 58.5%	< 0.01	Yes	+ 65.4%	< 0.01	Yes
CD11b	+ 13.9 %	< 0.01	Yes	+ 32.9 %	< 0.01	Yes
CD16	+ 0.9%	0.68	No	+ 3.9%	0.14	No
CD44	- 17.6%	< 0.01	Yes	+ 3.6%	0.31	N/A
L-selectin	- 31.5%	< 0.01	Yes	- 47.7%	< 0.01	Yes

1. Data presented are mean fluorescence intensity calculated as a percentage of baseline (media alone).
2. See References cited in Table 5.2.]

Table 5.2: References for ROS, CD11b, CD16, L-selectin and CD44 as markers of neutrophil and eosinophil activation.

	Expected Increase or Decrease	Activation Hypothesis References[1]
ROS	Increase	Brown and Roth, 1991;Leino and Paape, 1993; Conejeros *et al.*, 2011; Winterbourn, Kettle and Hampton, 2016;Sample and Czuprynski, 1990;Kowanko and Ferrante, 1987; Chaves *et al.*, 1996; Yukawa *et al.*, 1990
CD11b	Increase	In't Veen *et al.*, 1998; Scanzano *et al.*, 2015; Margaryan *et al.*, 2017; Brandau *et al.*, 2011; Casanova-Acebes *et al.* 2013;Trabold, Gruber and Fröhlich, 2007
CD16	Increase	Davoine *et al.*, 2002; Millrud *et al.*, 2017; Brandau *et al.*, 2011; Pillay *et al.*, 2012
CD44	Decrease	Wang *et al.*, 2002; Campanero *et al.*, 1991
L-selectin	Decrease	Tak *et al.*, 2017; Casanova-Acebes *et al.*, 2013; Ivetic, Green and Hart, 2019; Trabold, Gruber and Fröhlich, 2007; Margaryan *et al.*, 2017; Hayashi, Means and Luster, 2003; Li *et al.*, 2006; Diez-Fraile *et al.*, 2003; Momose *et al*., 1999

5.3 ADRENERGIC RECEPTOR FUNCTION IN BOVINE NEUTROPHILS AND EOSINOPHILS

5.3.1 Effects of Adrenergic Agonists on iROS Production in Resting and Activated Bovine Neutrophils and Eosinophils

Five adrenergic agonists were tested to determine if they influenced resting and activated neutrophils and eosinophils. These compounds included the endogenous agonists E and NE and synthetic agonists with known AR specificity. To address the question whether all families of ARs were possibly functional in eosinophils and neutrophils, compounds were selected with specificity for each AR family: phenylephrine (α1-AR agonist); dexmedetomidine (α2-AR agonist), and isoproterenol (β-AR agonist). The following data were normalized by expressing responses of cells from individual animals as a percentage of the response of isogenic cells cultured in media alone or activated by BoZ + rBoIFNγ in the absence of adrenergic agonist.

This normalization was used to minimize the effect of inter-animal variation in neutrophil and eosinophil responses to adrenergic agonists.

5.3.1.1 Intracellular Reactive Oxygen Species (iROS)

There is previous evidence that adrenergic agonists and antagonists can modulate ROS levels in bovine PMNs (LaBranche, Ehrich and Eyre, 2010). The production of iROS is an important effector mechanism for killing phagocytosed microbes and increased iROS production is also considered a sign of activation in both neutrophils (Winterbourn, Kettle and Hampton, 2016) and eosinophils (Yukawa *et al.*, 1990; Silveira *et al.*, 2019). Therefore, modulating iROS activity during a stress response may be very important in altering host-pathogen interactions.

All five adrenergic agonists tested altered iROS production in resting bovine neutrophils (Figure 5.17). Both physiological agonists, E and NE significantly ($p < 0.05$) increased iROS production. Norepinephrine significantly ($p < 0.05$) increased iROS production at all doses tested but the response was less consistent with E despite a numerical increase in iROS production at all doses tested.

Phenylephrine, a non-selective α1-AR agonist, consistently suppressed iROS activity in resting neutrophils but this reduction was only significant ($p < 0.05$) at a dose of 1 nM. Dexmedetomidine, a non-selective α2-AR agonist, also induced a consistent decrease in iROS production that was significant ($p < 0.01$) at a dose of 1 nM. Isoproterenol, the non-selective β-AR agonist, also inhibited iROS production with a significant ($p < 0.05$) effect at a concentration of 10 nM. At agonist concentrations of 0.1 and 100 nM, there were significant ($p < 0.05$) differences in the iROS response to E and NE relative to the synthetic adrenergic agonists phenylephrine, dexmedetomidine and isoproterenol. At the intervening doses of 1 and 10 nM,

this difference in iROS production when comparing E and NE with the synthetic adrenergic agonists was not always consistent but the same general trend was observed. Synthetic AR agonists, which target an individual AR family, suppressed iROS activity but both E and NE increased iROS activity in resting neutrophils. This apparent contradiction in the regulation of iROS activity by ARs was unexpected and suggests E and NE regulate iROS activity by signaling through more than just one AR family.

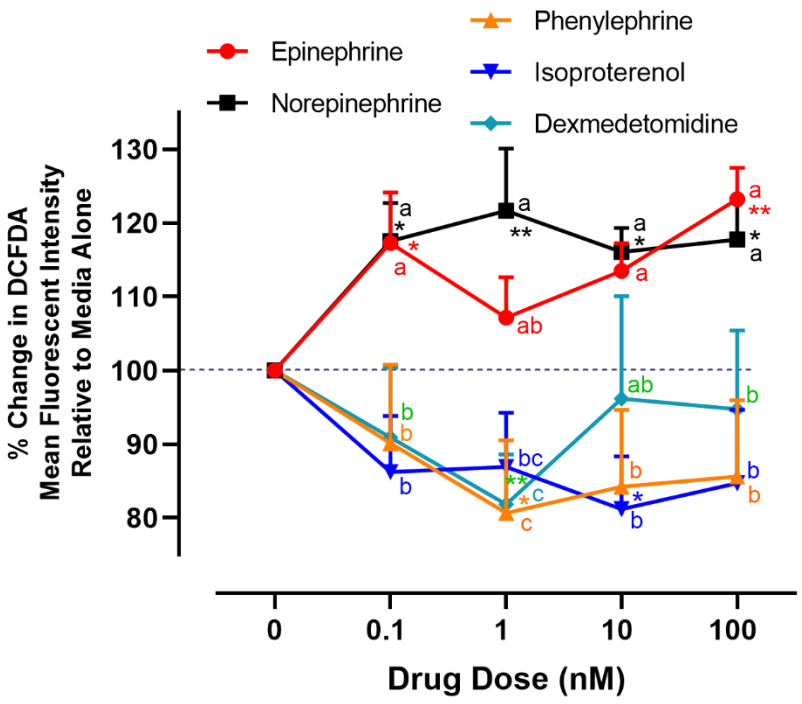


Figure 5.17: Effect of adrenergic agonists on iROS activity in resting bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of DCFDA mean fluorescence intensity (MFI) calculated as a percentage of DCFDA MFI intensity for neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare DCFDA MFI among different doses of each AR agonist and among different AR agonists at each dose. Significant differences in the expression of DCFDA MFI among treatments are identified by different letters (a, b, c).

Significant changes in DCFDA MFI relative to baseline are indicated for each treatment as * ($p < 0.05$) and ** ($p < 0.01$).

Among the five adrenergic agonists evaluated, only two agonists significantly ($p < 0.05$) altered iROS production following neutrophil activation by BoZ + IFNγ co-stimulation (Figure 5.18). NE induced a numerical increase in iROS production at all doses tested and this effect was significant ($p < 0.05$) at 1 nM, 10 nM, and 100 nM. This response was similar to that observed with resting neutrophils. In contrast, E had no significant effect on iROS production in activated neutrophils. Thus, a marked difference in E and NE activity was apparent when evaluating activated neutrophils. Dexmedetomidine, the non-selective α2-AR agonist, was the only synthetic agonist to significantly ($p < 0.05$) reduce iROS activity in activated neutrophils at doses of 1 nM and 10 nM despite a numerical decrease in iROS activity at all doses tested. This response is consistent with that observed with resting neutrophils. In contrast, both the non-selective α1-AR agonist (Phenylephrine) and β-AR agonist (Isoproterenol) failed to significantly alter iROS activity in activated neutrophils. There was, however, a numerical decrease in iROS production at both 1 and 10 nM doses for both AR agonists. There was a significant ($p < 0.05$) difference in iROS activity when comparing NE treatment with all synthetic adrenergic agonists at doses of 1 nM and higher. There was also a significant ($p < 0.05$) difference in iROS activity between NE and E treatments at 100 nM. These data indicate that regulation of iROS activity by adrenergic agonists can differ between resting and activated neutrophils and confirm NE stimulation of activated neutrophils is not replicated by a synthetic adrenergic agonist specifically targeting one AR family.

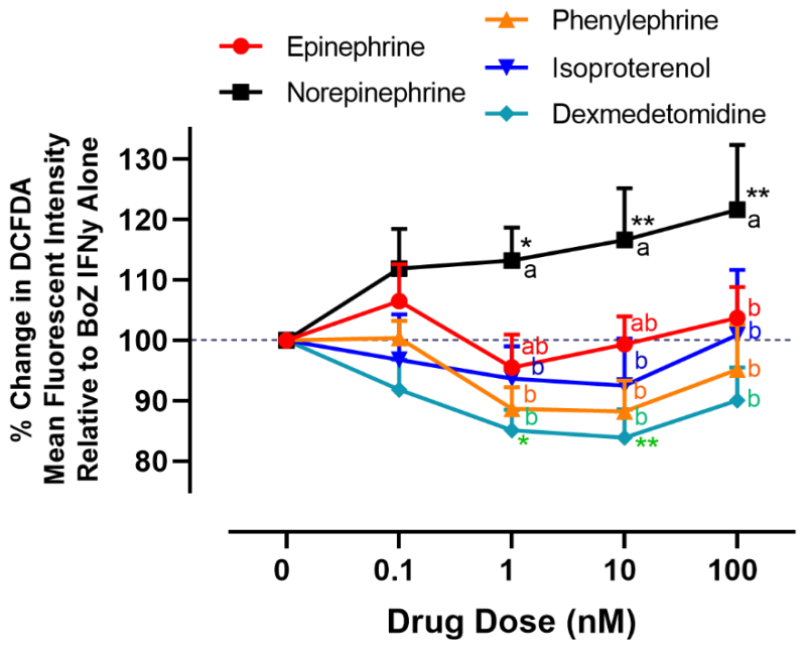


Figure 5.18: Effect of adrenergic agonists on iROS activity in activated bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of DCFDA mean fluorescence intensity (MFI) calculated as a percentage of DCFDA MFI intensity for BoZ rBoIFNγ activated neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare DCFDA MFI at different doses of each AR agonist and among AR agonists at the same dose. Significant differences in DCFDA MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences in DCFDA MFI are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Neither E nor NE had a significant effect on iROS activity in resting eosinophils but all three synthetic AR family specific agonists significantly reduced iROS activity (Figure 5.19). Phenylephrine, an α1-AR selective agonist, significantly ($p < 0.01$) reduced iROS expression at a concentration of 1, 10, and 100 nM. Dexmedetomidine, an α2-AR selective agonist, significantly ($p < 0.01$) reduced iROS expression at a concentration of 1 nM and isoproterenol, a β-AR

agonist, significantly ($p < 0.01$) reduced iROS at all doses tested. There were no significant differences in iROS suppression when comparing among the three synthetic adrenergic agonists but the response to isoproterenol was significantly different ($p < 0.01$) from E and NE at all doses tested. Significant differences between the synthetic agonists, dexmedetomidine and phenylephrine, and either E or NE were less consistent and only observed at doses between 1 to 100 nM. Thus, modulation of iROS activity in resting eosinophils was very different from resting neutrophils for E and NE but a similar pattern of suppressed iROS was observed with the three synthetic agonists.

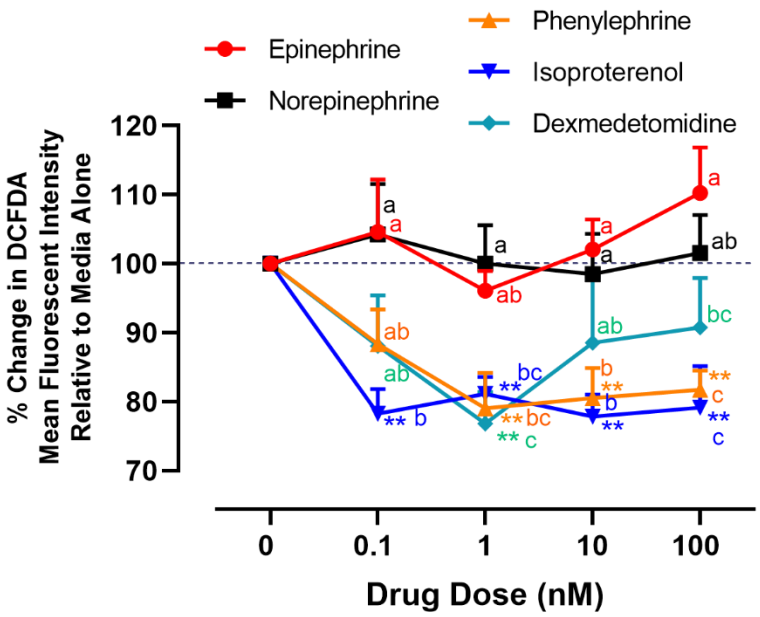


Figure 5.19: Effect of adrenergic agonists on iROS activity in resting bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of DCFDA mean fluorescence intensity (MFI) calculated as a percentage of DCFDA MFI intensity for eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare DCFDA MFI at different doses of each AR agonist and among AR agonists at the same dose. Significant differences in DCFDA

MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Similar to resting eosinophils, iROS levels were not significantly changed when activated eosinophils were incubated with E and NE (Figure 5.20). In contrast, two of the three synthetic AR family specific agonists (phenylephrine and isoproterenol) significantly decreased iROS activity in activated eosinophils. Phenylephrine, an α1-AR selective agonist, significantly reduced ($p < 0.05$) iROS expression at concentrations of 1, 10, and 100 nM, similar to what was observed with resting eosinophils. Isoproterenol, the β-AR agonist, also significantly ($p < 0.05$) reduced iROS at concentrations of 1, 10, and 100 nM. Dexmedetomidine, the α2-AR agonist, had no significant effect on iROS activity in activated eosinophils despite having a significant effect on resting eosinophils. At a dose of both 1 nM and 10 nM, both phenylephrine and isoproterenol induced significantly ($p < 0.05$) lower iROS activity relative to NE. Thus, eosinophil activation did not change the suppression of iROS by α1-AR and β-AR agonists. However, activated neutrophils (Figure 5.18) and activated eosinophils (Figure 5.20) displayed differences in their response to NE.

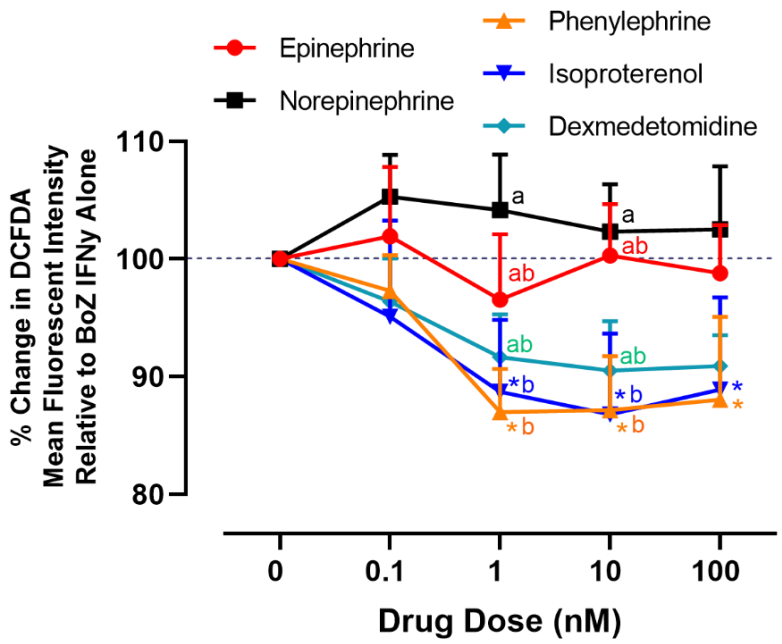


Figure 5.20: Effect of adrenergic agonists on iROS production in activated bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of DCFDA mean fluorescence intensity (MFI) calculated as a percentage of DCFDA MFI intensity for BoZ + rBoIFNγ activated eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare DCFDA MFI among doses of each AR agonist and among different AR agonists at the same dose. Significant differences in DCFDA MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

5.3.2 Effects of Adrenergic Agonists on Adhesion Molecule Expression of Resting and Activated Bovine Neutrophils and Eosinophils

The expression of adhesion molecules on neutrophils and eosinophils plays a critical role in cell recruitment and retention at sites of inflammation. They can also be used as markers for PMN activation (Walker *et al.*, 1993; Mengelers *et al.*, 1994; In't Veen *et al.*, 1998; Wang *et al.*,

2002; Lee *et al.*, 2007; Casanova-Acebes *et al.*, 2013; Tak *et al.*, 2017; Cassetta *et al.*, 2019; Ivetic, Green and Hart, 2019). Stress has also been shown to alter neutrophil and eosinophil recruitment to tissues and their abundance in the blood in cattle (Hickey, Drennan and Earley, 2003; Yagi *et al.*, 2004; Ishizaki and Kariya, 2010; Hodgson *et al.*, 2012; O'Loughlin *et al.*, 2011). Furthermore, *in vivo* studies have shown adrenergic agonists alter PMN recruitment to sites of infection such as wounds in mice (Gosain, Gamelli and DiPietro, 2009a; Kim *et al.*, 2014). Therefore, I investigated whether adrenergic agonists modulated the expression of key adhesion molecules, CD11b, L-selectin, and CD44, on both resting and activated neutrophils and eosinophils.

5.3.2.1 CD11b

CD11b plays a role in neutrophil adhesion to vascular endothelium and Fc receptor mediated degranulation (Coxon *et al.*, 1996; Tang *et al.*, 1997; Ince, Weber and Scheiermann, 2019). Increased CD11b expression is indicative of neutrophil and eosinophil activation and increased responsiveness to pathogens such as bacteria and environmental fungi (Walker *et al.*, 1993; In't Veen *et al.*, 1998; Casanova-Acebes *et al.*, 2013; Cassetta *et al.*, 2019) and adrenergic agonists are reported to increase CD11b expression on PMNs (Trabold, Gruber and Fröhlich, 2007; Kim *et al.*, 2014; Scanzano *et al.*, 2015; Margaryan *et al.*, 2017). Therefore, the capacity of adrenergic agonists to modulate CD11b expression on resting and activated bovine neutrophils and eosinophils was investigated to determine if these two populations displayed similar responses.

All adrenergic agonists tested increased the expression of CD11b on resting neutrophils (Figure 5.21). The physiological agonists E and NE significantly ($p < 0.05$) increase CD11b expression at 1, 10, and 100 nM concentrations. The α1-AR agonist phenylephrine also

significantly ($p < 0.05$) increases CD11b expression but only at the highest dose of 100 nM. This suggests the α1-AR family may contribute to the effect of E and NE on CD11b expression in bovine neutrophils but at 10 nM, NE induced a significantly ($p < 0.05$) greater CD11b response than any of the synthetic AR family specific agonists. Thus, no one family of ARs is implicated as being primarily responsible for mediating the effects of E and NE on CD11b expression. All synthetic adrenergic agonists did, however, induce a numerical if not significant increase in CD11b expression suggesting multiple ARs may be implicated in mediating the effect of E and NE.

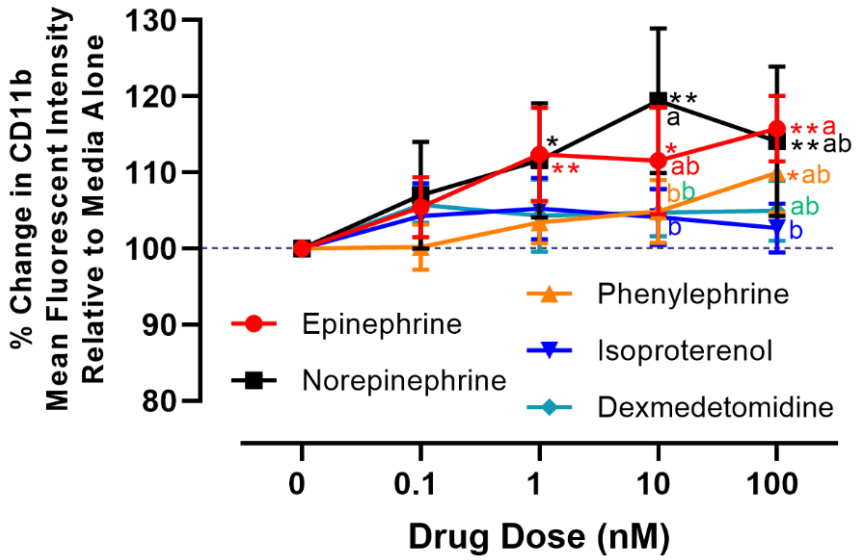


Figure 5.21: Effect of adrenergic agonists on CD11b expression on resting bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of CD11b mean fluorescence intensity (MFI) calculated as a percentage of CD11b intensity for neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD11b MFI among doses of each AR agonist and among AR agonists at the same dose. Significant differences in CD11b MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Adrenergic agonists also increased CD11b expression on neutrophils activated with BoZ + rBoIFNγ (Figure 5.22). Epinephrine and norepinephrine significantly ($p < 0.05$) increased CD11b expression only at the highest dose tested. Isoproterenol, the β-AR agonist, significantly ($p < 0.01$) increased CD11b expression only at 0.1 nM but consistently increased CD11b expression at all doses evaluated. Furthermore, increased CD11b expression induced by isoproterenol at 0.1 nM was significantly ($p < 0.01$) greater than that observed with E but not NE. At 100 nM, NE, phenylephrine, and dexmedetomidine significantly ($p < 0.05$) increased CD11b expression but there was no difference in this response when comparing among agonists. Thus, the capacity of adrenergic agonists to increase CD11b was observed with both resting and activated neutrophils but the response varied, depending on the specific agonist. For example, E significantly increased CD11b expression in resting (Figure 5.21) but not activated (Figure 5.22) neutrophils.

Similar to neutrophils, resting bovine eosinophils also responded to adrenergic agonists with an increase in CD11b expression (Figure 5.22). Both E and NE significantly ($p < 0.01$) increased CD11b expression at doses of 1 to 100 nM. In contrast, the synthetic adrenergic agonists induced a numerical increase in CD11b expression, but this effect was only significant ($p < 0.01$) for phenylephrine at a dose of 100 nM. At a dose of 10 nM, NE induced a significantly ($p < 0.05$) greater increase in CD11b expression than any of the synthetic agonists targeting individual AR families. This observation supports the conclusion that the effect of both E and NE cannot be ascribed to signaling through a single AR family.

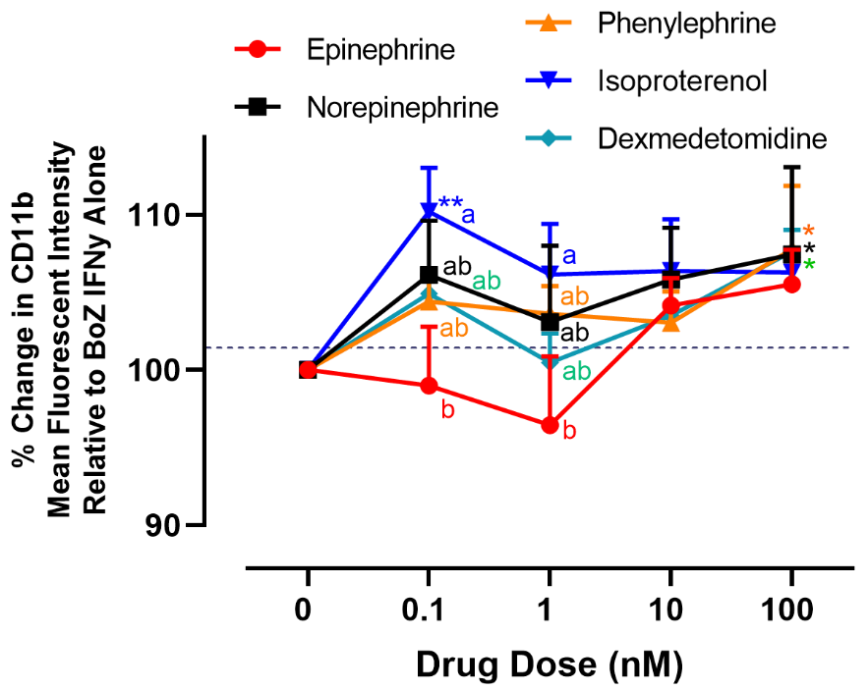


Figure 5.22: Effect of adrenergic agonists on CD11b expression on activated bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of CD11b mean fluorescence intensity (MFI) calculated as a percentage of CD11b intensity for neutrophils activated with BoZ + rBoIFNγ in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD11b MFI among doses of each AR agonist and among AR agonists at the same dose. Significant differences in CD11b MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent effects relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

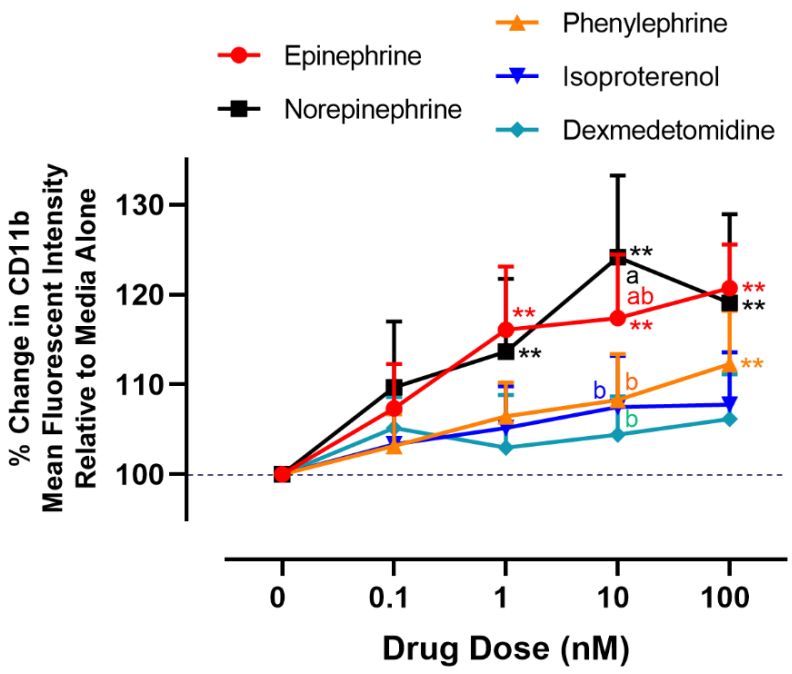


Figure 5.23: Effect of adrenergic agonists on CD11b expression on resting bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD11b mean fluorescence intensity (MFI) calculated as a percentage of CD11b intensity for eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD11b MFI among doses of each AR agonist and among AR agonists at the same dose. Significant differences in CD11b MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent effects relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

With activated eosinophils, all adrenergic agonists induced a numerical increase in CD11b expression but a significant effect was only observed at a few specific doses (Figure 5.24). At a dose of 0.1 nM, the β-AR agonist isoproterenol induced a significant ($p < 0.01$) increase in CD11b expression. At a dose of 100 nM, the α1-AR agonist phenylephrine and α2-AR agonist dexmedetomidine also induced significant ($p < 0.01$) increases in CD11b expression. Unlike resting eosinophils, neither E nor NE induced significant increases in CD11b expression

but there was a numerical increase in CD11b MFI at most doses evaluated. Thus, the pattern of adrenergic agonist activity was similar in both resting and activated eosinophils.

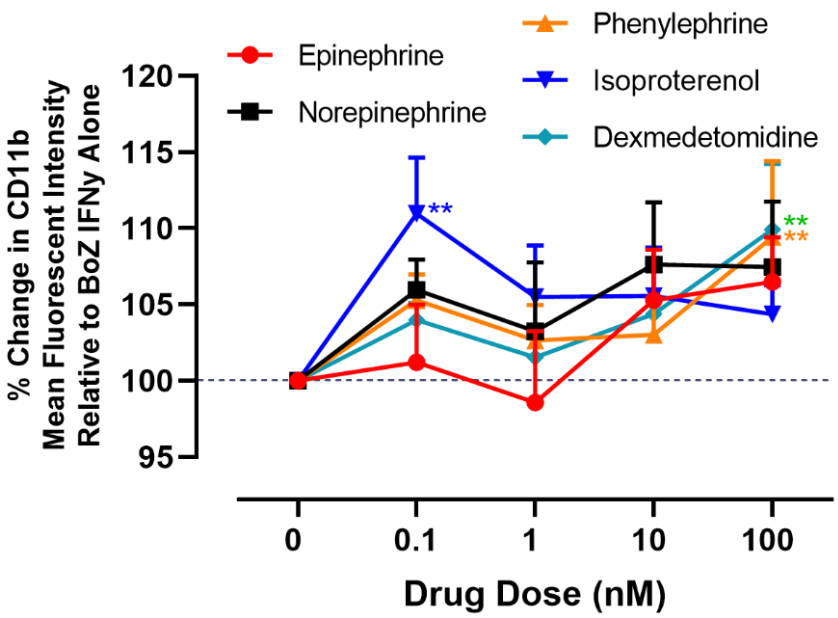


Figure 5.24: Effect of adrenergic agonists on CD11b expression on activated bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD11b mean fluorescence intensity (MFI) calculated as a percentage of CD11b intensity for eosinophils activated with BoZ + rBoIFNγ in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD11b MFI among doses of each AR agonist and among AR agonists at the same dose. No significant differences in CD11b MFI was observed among treatments but significant dose-dependent differences relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

5.3.2.2 L-selectin

L-selectin is an adhesion molecule which is "shed" during activation and maturation of neutrophils and eosinophils (Mengelers *et al.*, 1994; Lee *et al.*, 2007; Casanova-Acebes *et al.*, 2013; Tak *et al.*, 2017; Ivetic, Green and Hart, 2019). Epinephrine and norepinephrine have been

reported to modulate L-selectin expression on human neutrophils (Trabold, Gruber and Fröhlich, 2007) but there are no reports that adrenergic agonists modulate L-selectin levels on bovine neutrophils or eosinophils. Therefore, the effects of adrenergic agonists on the expression of L-selectin on both resting and activated bovine neutrophils and eosinophils was investigated.

All adrenergic agonists evaluated significantly ($p < 0.01$) reduced L-selectin expression on resting bovine neutrophils at all doses tested and the magnitude of this response was similar at 0.1 nM and 100 nM for each agonist (Figure 5.25). The β-AR agonist isoproterenol consistently induced the greatest reduction in L-selectin expression and the magnitude of this response at 0.1 nM was significantly ($p < 0.05$) greater than either of the physiological ligands, E and NE. However, at higher doses, no significant differences were observed among the five adrenergic agonists. These results confirm adrenergic agonists consistently induce rapid shedding of L-selectin on resting bovine neutrophils and all three AR families can mediate this effect.

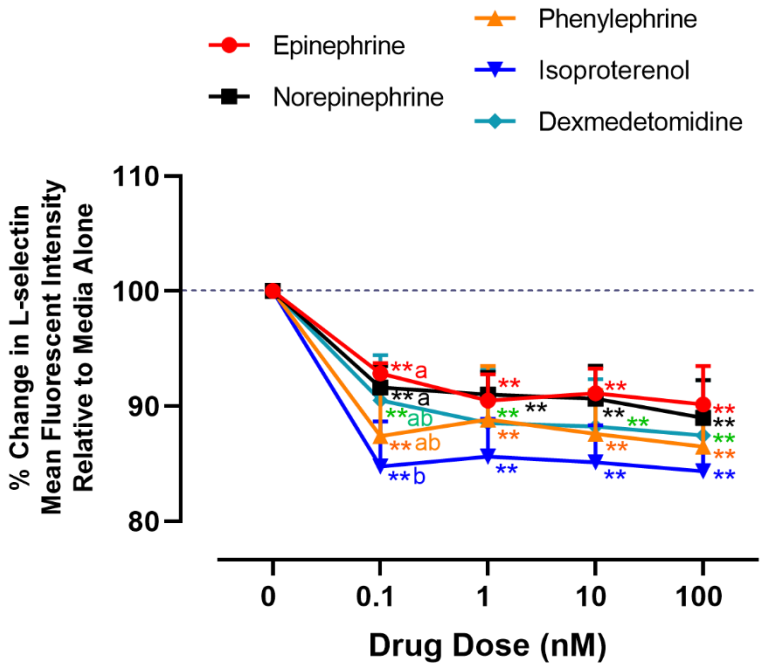


Figure 5.25: Effect of adrenergic agonists on L-selectin levels on resting bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of L-selectin mean fluorescence intensity (MFI) calculated as a percentage of L-selectin MFI intensity for resting neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare L-selectin MFI among doses of each AR agonist and among AR agonists at the same dose. Significant differences in L-selectin MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences within each treatment relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Activation of bovine neutrophils with BoZ + rBoIFNγ results in a 50-60% reduction in L-selectin (Figure 5.16) and adrenergic agonists had a less marked effect on L-selectin expression on activated bovine neutrophils (Figure 5.26). For most agonists, except for E, there was a numerical decrease in L-selectin expression that was dose-dependent. Dexmedetomidine, the α2 agonist, significantly ($p < 0.05$) reduced L-selectin at doses of 1, 10, and 100 nM. At 100 nM, dexmedetomidine significantly ($p < 0.05$) reduced L-selectin levels relative to the physiological agonists E and NE. Isoproterenol, the β-AR agonist, also significantly ($p < 0.05$) reduce L-selectin expression a at 10 nM. Overall, the effect of adrenergic agonists on L-selectin shedding was much less pronounced in activated (Figure 5.26) versus resting (Figure 5.25) bovine neutrophils. This limited effect by adrenergic agonists may simply be a consequence of the substantial loss of L-selectin when neutrophils are activated with BoZ + rBoIFNγ (Figure 5.16).

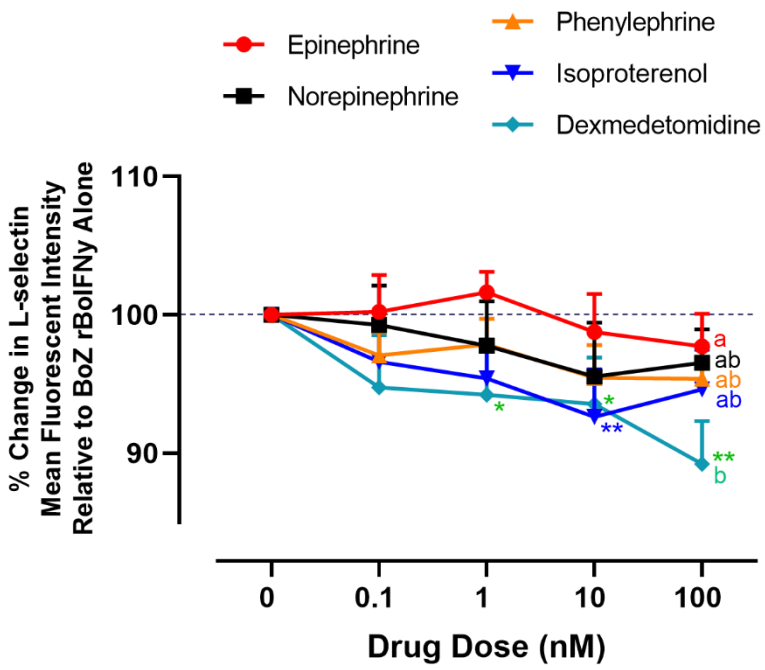


Figure 5.26: Effect of adrenergic agonists on L-selectin levels on activated bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of L-selectin mean fluorescence intensity (MFI) calculated as a percentage of L-selectin MFI intensity for BoZ + rBoIFNγ activated neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare L-selectin MFI among doses for each AR agonist and among AR agonists at the same dose. Significant differences in L-selectin MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Adrenergic agonists both increased and decreased L-selectin levels on resting eosinophils (Figure 5.27). Surprisingly, NE significantly ($p < 0.01$) increased L-selectin levels at doses of 0.1 and 1 nM and at these doses L-selectin levels on NE treated eosinophils were significantly ($p < 0.05$) greater than on eosinophils treated with all other adrenergic agonists. In contrast, phenylephrine, the α1-AR agonist, significantly ($p < 0.05$) reduced L-selectin expression at

doses of 0.1, 1, and 10 nM. Isoproterenol, the β-AR agonist, also significantly ($p < 0.01$) reduced L-selectin expression at 10 and 100 nM doses and at 10 nM isoproterenol induced significantly ($p < 0.05$) less L-selectin than both NE and dexmedetomidine treated eosinophils. Dexmedetomidine, the a2-AR agonist, and E had no significant effect on L-selectin levels on resting eosinophils. Thus, when analyzing L-selectin levels on resting eosinophils (Figure 5.27) these cells displayed a much more varied response to adrenergic agonists than neutrophils (Figure 5.25). Furthermore, the L-selectin response on resting eosinophils to synthetic adrenergic agonists confirmed both α1- and β-ARs were functional but signaling by these AR families could not account for NE activity. Finally, the contradictory effect of NE on L-selectin levels in resting neutrophils and eosinophils further demonstrates the importance of separately analyzing the responses of these two PMN subpopulations.

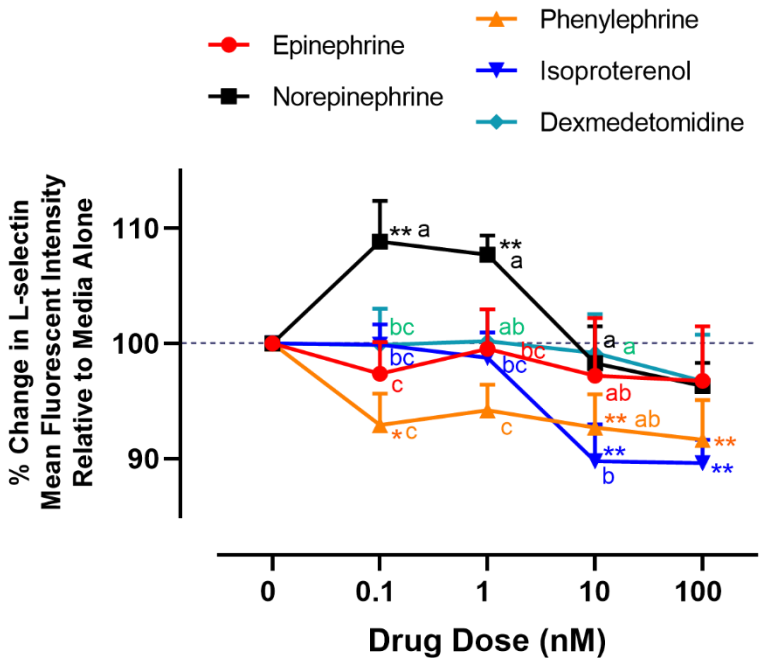


Figure 5.27: Effect of adrenergic agonists on L-selectin levels on resting bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of L-selectin mean fluorescence intensity (MFI)

calculated as a percentage of L-selectin MFI intensity for eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare L-selectin MFI among doses for each AR agonist and among AR agonists at the same dose. Significant differences in L-selectin MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences within individual treatments relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

As observed with neutrophils, activation of eosinophils with BoZ + rBoIFNγ resulted in a marked reduction in L-selectin (Figure 5.16). None of the five adrenergic agonists evaluated, however, induced a further significant change in L-selectin levels on activated eosinophils with the exception of the α2-AR agonist dexmedetomidine ($p < 0.01$) at a dose of 100 nM, (Figure 5.28). At this dose, the reduction in L-selectin by dexmedetomidine was significantly ($p < 0.05$) different when compared to E but none of the other AR agonists. This apparent lack of an effect by AR agonists on L-selectin levels on activated eosinophils was similar to the response observed with activated neutrophils.

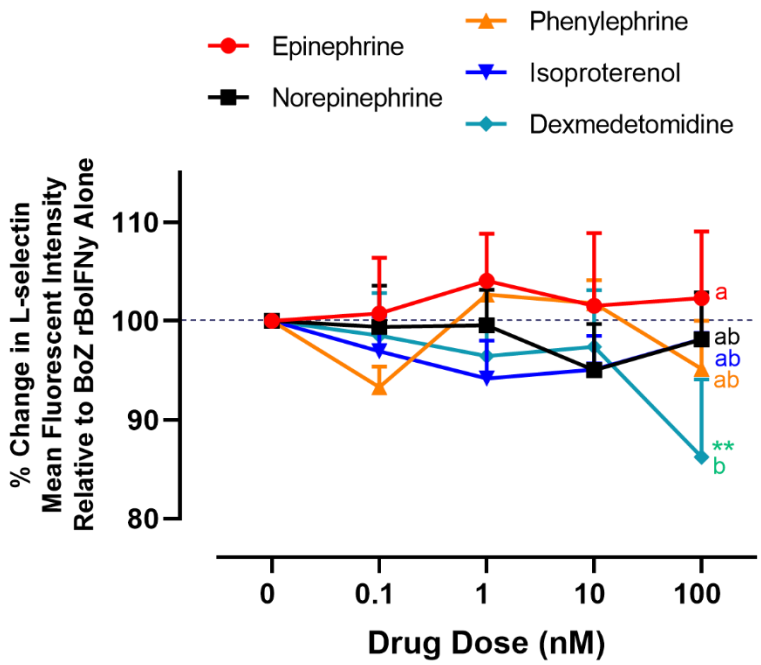


Figure 5.28: Effect of adrenergic agonists on L-selectin levels on activated bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of L-selectin mean fluorescence intensity (MFI) calculated as a percentage of L-selectin MFI intensity for activated eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to analyze L-selectin MFI among doses for each AR agonist and among AR agonists at the same dose. Significant differences in L-selectin MFI among treatments are identified by different letters (a, b) and there were no significant dose-dependent differences when comparing within treatment relative to baseline.

5.3.2.3 CD44

CD44 is a receptor that augments cell adhesion to and monitoring of hyaluronan in the extracellular matrix (Ponta, Sherman and Herrlich, 2003). Down-regulation of the CD44 receptor by cells such as neutrophils has been shown to increase their migration by reducing cell adhesion to hyaluronan (Wang *et al*., 2002). CD44 has also been found to be integral to allergic responses

by eosinophils, on the other hand, meaning decreased expression may inhibit function (Katoh *et al.*, 2003). Therefore, determining whether adrenergic agonists modulate CD44 expression in either resting or activated neutrophils and eosinophils may provide insight into the effect of stress on recruitment of these cells to sites of inflammation.

Although neutrophils express a much higher level of CD44 than eosinophils (Figure 5.15) none of the adrenergic agonists analyzed had a significant effect on CD44 expression on either resting (Figure 5.29) or BoZ + rBoIFNγ activated (Figure 5.30) bovine neutrophils.

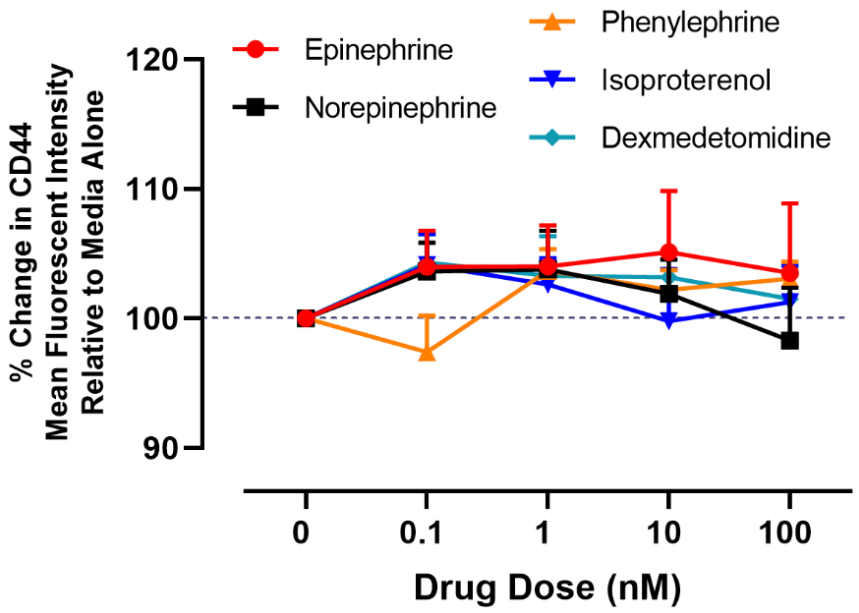


Figure 5.29: Effect of adrenergic agonists on CD44 expression on resting bovine neutrophils Data presented are mean ± SEM (n = 5 cattle) of CD44 mean fluorescence intensity (MFI) calculated as a percentage of CD44 MFI intensity for neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD44 MFI among doses of each AR agonist and among AR agonists at the same dose. No significant differences in CD44 MFI among treatments were identified and no significant dose-dependent differences were identified within individual treatments.

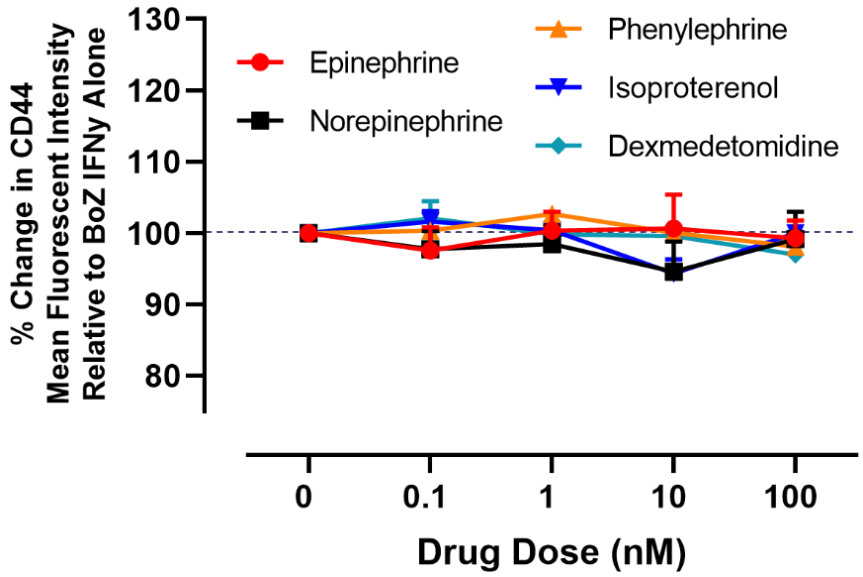


Figure 5.30: Effect of adrenergic agonists on CD44 expression on activated bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of CD44 mean fluorescence intensity (MFI) calculated as a percentage of CD44 MFI intensity for BoZ + rBoIFNγ activated neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD44 MFI among doses of each AR agonist and among AR agonists at each dose. No significant differences in CD44 MFI were identified among treatments and no significant dose-dependent differences were observed within individual treatments.

Endogenous AR agonists E and NE significantly increased CD44 expression on resting bovine eosinophils (Figure 5.31). At a dose of 1 nM, NE significantly ($p < 0.01$) increased CD44 expression, but this effect was not significantly different when compared to other AR agonists. Similarly, at concentrations of 1 and 10 nM, E significantly ($p < 0.05$) increased CD44 expression but again this effect was also not significantly different from other AR agonists. The synthetic AR agonists followed a similar trend with numerical increases in CD44 expression that were not, however, significantly different from baseline levels. These observations further highlight differences in the responses to AR agonists by resting neutrophil and eosinophils.

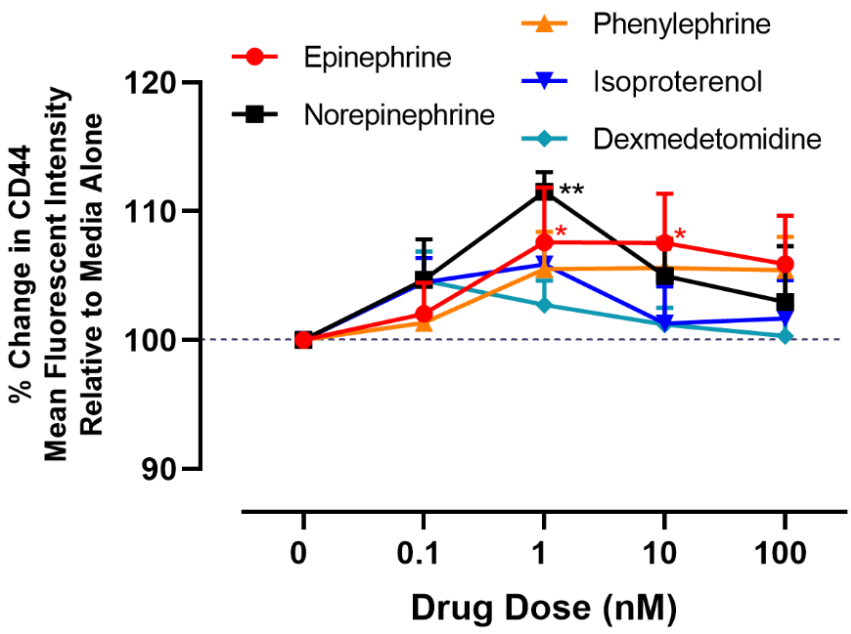


Figure 5.31: Effect of adrenergic agonists on CD44 expression on resting bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD44 mean fluorescence intensity (MFI) calculated as a percentage of CD44 MFI intensity for resting eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD44 MFI among doses for each AR agonist and among AR agonists at the same dose. There were no significant differences when comparing CD44 MFI among treatments but significant dose-dependent differences within individual treatments relative to baseline are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

Activation of bovine eosinophils by BoZ + rBoIFNγ does not significantly alter CD44 levels (Figure 5.16). Treatment of activated eosinophils with AR agonists also had no significant effect on CD44 expression with the exception of E which at a dose of 1 nM induced a significantly ($p < 0.05$) reduction in CD44 (Figure 5.32). The synthetic AR agonists induced numerical but not significant dose-dependent increases in CD44 expression but 0.1 nM dexmedetomidine, the α2-AR agonist, induced significantly ($p < 0.05$) higher CD44 expression

than NE. Furthermore, at a dose of 1 nM, dexmedetomidine, phenylephrine, and isoproterenol induced significantly ($p < 0.05$) higher CD44 expression when compared to E. These results imply there may be some differences in eosinophil activation by endogenous and synthetic AR agonists. However, it should be noted that these differences in CD44 expression were small.

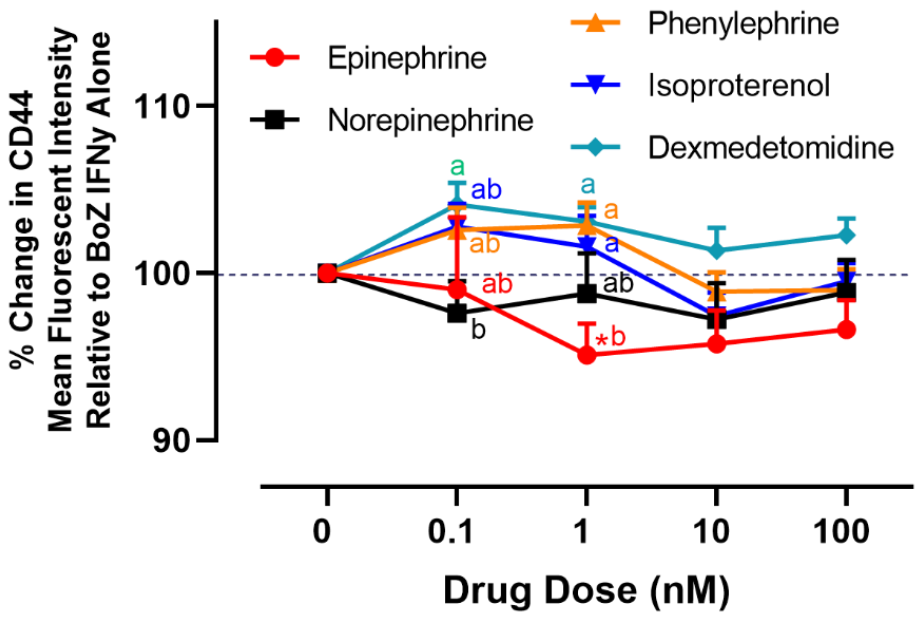


Figure 5.32: Effect of adrenergic agonists on CD44 expression on activated bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD44 mean fluorescence intensity (MFI) calculated as a percentage of CD44 MFI intensity for activated eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD44 MFI among doses for each AR agonist and among AR agonists at the same dose. Significant differences in the expression of CD44 MFI among treatments are identified by different letters (a, b, c) and significant dose-dependent differences within treatment relative to baseline are indicated as * ($p < 0.05$).

5.3.3 Effects of Adrenergic Agonists on Expression of the Fc Receptor CD16 on Resting and Activated Bovine Neutrophils and Eosinophils

CD16 is an Fc receptor that is considered a marker of both neutrophil and eosinophil activation and maturation (Huizinga *et al.*, 1990; Kimberly *et al.*, 1990; Davoine *et al.*, 2002; Pillay *et al.*, 2012; Millrud *et al.*, 2017). It has been associated with multiple PMN activation responses such as oxidative burst and phagocytosis (Huizinga *et al.*, 1990; Kimberly *et al.*, 1990). Thus, modulation of CD16 expression by AR agonists has implications for altering a wide array of neutrophil and eosinophil responses during a stress response.

5.3.3.1 CD16

CD16 expression (average MFI: 3.75) was detectable on approximately 30% of resting bovine neutrophils and treatment with the 5 AR agonists did not significantly alter CD16 expression (Figure 5.33). The one exception was a small but significant ($p < 0.05$) reduction in CD16 expression when resting neutrophils were treated with NE. This response to NE was not significant when compared to other AR agonists.

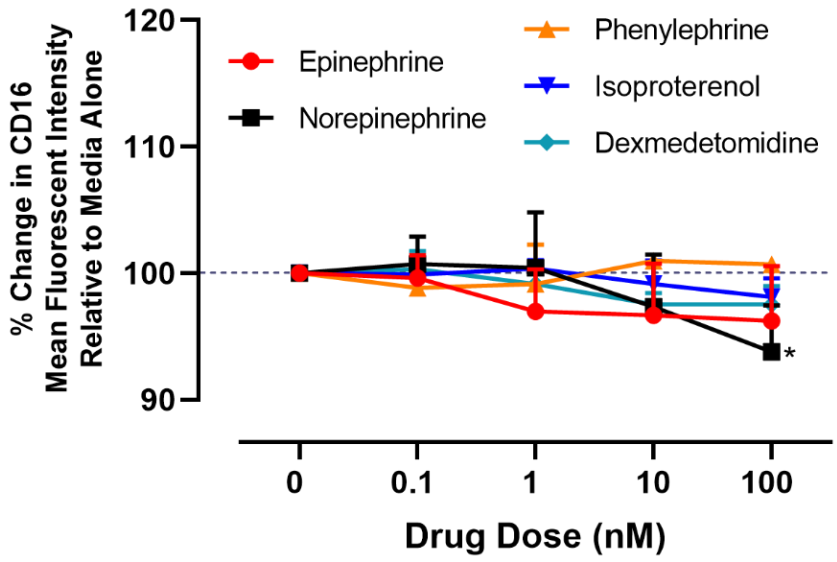


Figure 5.33: Effect of adrenergic agonists on CD16 expression on resting bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of CD16 mean fluorescence intensity (MFI) calculated as a percentage of CD16 MFI intensity for resting neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD16 MFI among doses for each AR agonist and among AR agonists at the same dose. No significant differences in CD16 MFI was observed among treatments and a significant dose-dependent difference in expression within individual treatment relative to baseline is indicated as * ($p < 0.05$).

Neutrophil activation with BoZ + rBoIFNγ did not significantly alter CD16 expression (Figure 5.16) and treatment of activated bovine neutrophils with all AR agonists, with the exception of isoproterenol, had no significant effect on CD16 expression (Figure 5.34). Treatment with 10 nM of the β-AR agonist isoproterenol induced a small but significant ($p < 0.05$) reduction in CD16 expression.

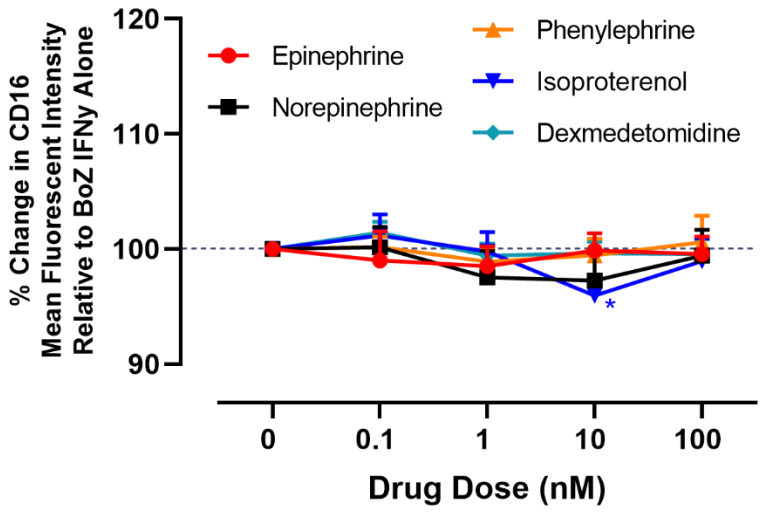


Figure 5.34: Effect of adrenergic agonists on CD16 expression on activated bovine neutrophils. Data presented are mean ± SEM (n = 5 cattle) of CD16 mean fluorescence intensity (MFI) calculated as a percentage of CD16 MFI intensity for BoZ + rBoIFNγ activated

neutrophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD16 MFI among doses of each AR agonist and among AR agonists at the same dose. There were no significant differences in CD16 MFI when comparing among treatments and significant dose-dependent differences within individual treatment relative to baseline are indicated as * ($p < 0.05$)

Less than one percent of resting eosinophils express a detectable level of CD16, but treatment with 0.1 and 1 nM NE significantly ($p < 0.01$) increased CD16 expression (Figure 5.35). This effect by NE was unique ($p < 0.05$) among the AR agonists evaluated at a dose of 1.0 nM. These results highlight that E and NE can have very different biological effects on a specific leukocyte subpopulation, such as eosinophils. Furthermore, the marked difference in NE modulation of CD16 on resting neutrophils (Figure 5.33) and eosinophils (Figure 5.35) provides evidence that differences in AR gene expression in these two populations (Figure 5.8) may influence responses to AR agonists.

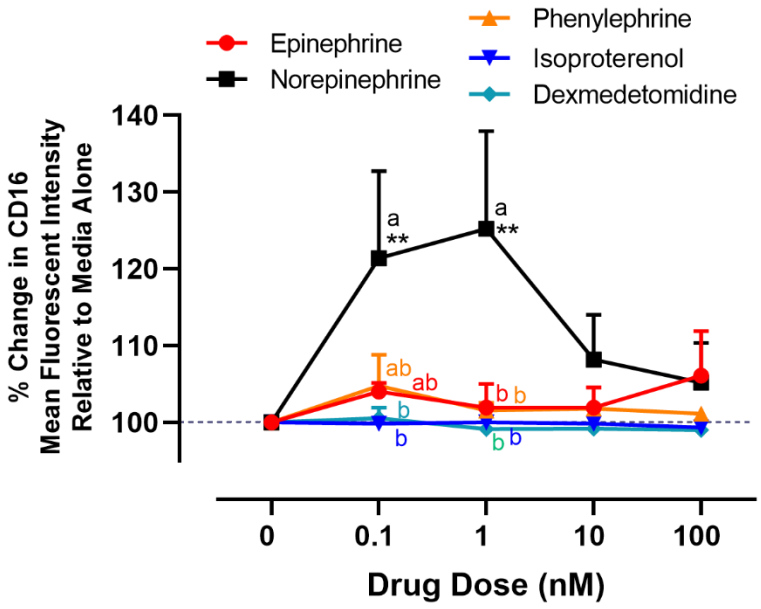


Figure 5.35: Effect of adrenergic agonists on CD16 expression on resting bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD16 mean fluorescence intensity (MFI) calculated as a percentage of CD16 MFI intensity for resting eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD16 MFI among doses for each AR agonist and among AR agonists at the same dose. Significant differences in CD16 MFI among treatments are identified by different letters (a, b) and significant dose-dependent differences within individual treatments relative to baseline are indicated as ** ($p < 0.01$).

Activation of eosinophils with BoZ + rBoIFNγ did not alter CD16 expression (Figure 5.16) and no significant alteration in CD16 expression on activated eosinophils was observed following treatment with adrenergic agonists (Figure 5.36). Activation of eosinophils may modulate either AR expression or signaling since NE no longer induce increased CD16 expression but rather induced a numerical decrease in CD16 expression.

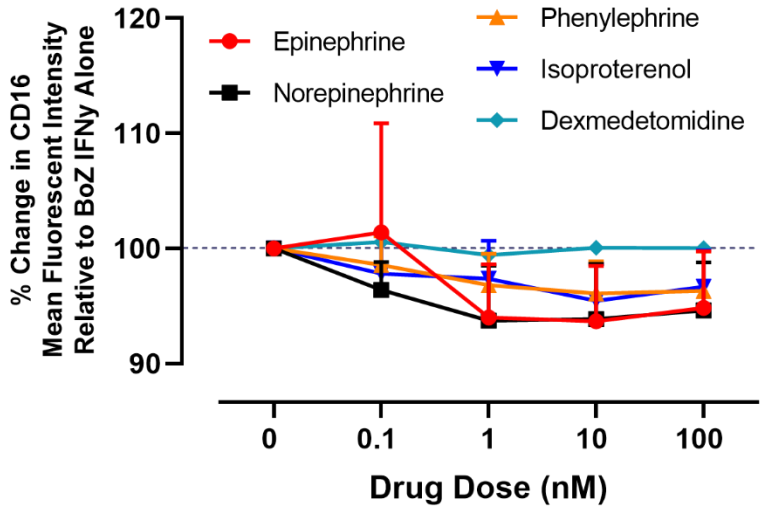


Figure 5.36: Effect of adrenergic agonists on CD16 expression on activated bovine eosinophils. Data presented are mean ± SEM (n = 5 cattle) of CD16 mean fluorescence intensity (MFI) calculated as a percentage of CD16 MFI intensity for BoZ + rBoIFNγ activated

eosinophils cultured in the absence of an adrenergic agonist. A two-way ANOVA was used to compare CD16 MFI among doses for each AR agonist and among AR agonists at each dose. No significant differences in CD16 MFI were observed among treatments and no significant dose-dependent differences were observed.

5.3.4 Summary: Adrenergic Receptor Function in Bovine Neutrophils and Eosinophils

Treatment with each of the five adrenergic agonists caused significant changes in the expression of one or more of the activation markers evaluated for both resting neutrophils and resting eosinophils (Table 5.2). This summary highlights a number of important observations when comparing adrenergic agonists within each population and when comparing the effect of individual adrenergic agonists between neutrophils and eosinophils.

Some primary effects observed in resting neutrophils included broad activation in the form of increased iROS, increased CD11b, and decreased L-selectin in response to the physiological agonists E and NE. The synthetic AR agonists phenylephrine (α1 agonist), dexmedetomidine (α2 agonist), and isoproterenol (β agonist) had a consistently suppressive effect on resting neutrophil iROS. However, phenylephrine treatment significantly increased CD11b, consistent with activation (In't Veen *et al.*, 1998; Brandau *et al*., 2011; Casanova-Acebes *et al*., 2013; Margaryan *et al*., 2017). Also, all synthetic AR agonists demonstrated L-selectin shedding, consistent with activation (Casanova-Acebes *et al.*, 2013; Tak *et al.*, 2017; Ivetic, Green and Hart, 2019). Based upon these results, adrenergic agonist treatment has many effects consistent with activation in resting neutrophils. However, the effect on iROS differs substantially when AR families are stimulated together rather than individually.

On resting eosinophils, several broad effects were also observed. E treatment increased the expression of CD11b and CD44, and NE increased CD11b, L-selectin, CD44, and CD16. Increased expression of CD11b, CD44 and CD16 on eosinophils is consistent with an activation phenotype (In't Veen et al., 1998; Davoine *et al*., 2002; Katoh.*et al*., 2003) (Table 5.1). In contrast to neutrophils, neither E nor NE affected iROS. For the synthetic AR agonists, in contrast, a strong suppressive effect on iROS was observed in eosinophils. This effect was similar to what was observed in neutrophils in response to the synthetic AR agonists. Phenylephrine (an α1-AR agonist) appeared to have a similar effect on both neutrophils and eosinophils, with the drug both increasing CD11b and decreasing L-selectin. Taken together, adrenergic agonists had several activating effects on resting eosinophils. However, agonists targeting individual AR families created a strong and consistent suppression of iROS. Overall, NE had the broadest and most consistent activation effect on resting neutrophils and eosinophils.

Table 5.3: Summary of adrenergic agonists' effects on resting neutrophils and eosinophils.

	Neutrophils					Eosinophils				
	iROS[1]	**CD11b[1]**	**L – selectin[1]**	**CD44[1]**	**CD16[1]**	**iROS[1]**	**CD11b[1]**	**L-selectin[1]**	**CD44[1]**	**CD16[1]**
Epinephrine	↑	↑[2]	↓[3]	-[4]	-	-	↑	-	↑	-
Norepinephrine	↑	↑	↓	-	↓	-	↑	↑	↑	↑
Phenylephrine	↓	↑	↓	-	-	↓	↑	↓	-	-
Dexmedetomidine	↓	-	↓	-	-	↓	-	-	-	-
Isoproterenol	↓	-	↓	-	-	↓	-	↓	-	-

1. Changes in iROS, CD11b, L-selectin, CD44, and CD16 expression used to determine if functional AR expressed in each activated PMN subpopulation and to determine if biological differences were apparent when comparing between PMN subpopulations.
2. Significant increases in expression are presented as green "↑"
3. Significant decreases in expression are presented as red "↓"

4. No significant change in expression is shown as "-".

Activation of neutrophils and eosinophils with BoZ + rBoIFNγ influenced many of the parameters (Figure 5.16) used to evaluate the effect of adrenergic agonists. These changes, such as increased L-selectin shedding, may have limited the ability to assess the effect of adrenergic agonists but for many of the parameters it was still possible to evaluate the effects of adrenergic agonists in activated neutrophils and eosinophils (Table 5.3). This evaluation provided insight into possible changes in AR function when PMNs are activated and many of the changes induced by adrenergic agonists highlight that there are marked differences when comparing activated versus resting cells.

Fewer changes were observed for activated neutrophils relative to resting neutrophils. A smaller number of effects, particularly, were observed for the markers iROS, L-selectin, and CD44. Similar to resting cells, NE induced increased iROS expression in activated neutrophils, and dexmedetomidine induced a decrease in iROS. However, there were no significant changes in iROS for the other three treatments. A decrease in L-selectin expression was observed for just two of the drug treatments: dexmedetomidine (an α2-AR agonist) and isoproterenol (a β-AR agonist). However, four of the five drugs (all treatments except E) induced increased CD11b expression in activated neutrophils. Overall, the effect of adrenergic agonists on activated neutrophils are primarily activating effects; however, these effects are smaller than those observed in resting neutrophils.

For activated eosinophils, smaller effects were also observed relative to their resting counterparts in response to adrenergic agonists. A significant suppressive effect on iROS was observed for just two of the three synthetic AR agonists: phenylephrine (an α1-AR agonist) and isoproterenol (a β-AR agonist). In resting eosinophils, CD11b expression increased in response

to both physiological agonists, E and NE, and phenylephrine. In contrast, activated eosinophils, did not respond to E and NE, but did respond to the synthetic AR agonists. One adrenergic agonist, dexmedetomidine, was observed decreasing L-selectin expression in activated eosinophils, whereas three agonists, NE, phenylephrine and isoproterenol, were observed decreasing L-selectin expression in resting eosinophils. This possibly demonstrates a greater sensitivity to activation in resting eosinophils. However, resting eosinophils increased their expression of CD44 in response to E treatment (indicative of activation), whereas activated eosinophils decreased their expression of CD44 in response to the same treatment (indicative of suppression) (Katoh *et al.*, 2003). Overall, the response in activated eosinophils appeared similar, but more muted relative to the response of resting eosinophils. However, some effects were notably different in activated cells.

Table 5.4: Summary of adrenergic agonists' effects on BoZ + rBoIFNγ activated neutrophils and eosinophils.

	Neutrophils					Eosinophils				
	iROS[1]	CD11b[1]	L-selectin[1]	CD44[1]	CD16[1]	iROS	CD11b	L-selectin	CD44	CD16
Epinephrine	-[2]	-	-	-	-	-	-	-	↓[3]	-
Norepinephrine	↑[4]	↑	-	-	-	-	-	-	-	-
Phenylephrine	-	↑	-	-	-	↓	↑	-	-	-
Dexmedetomidine	↓	↑	↓	-	-	-	↑	↓	-	-
Isoproterenol	-	↑	↓	-	↓	↓	↑	-	-	-

1. Changes in iROS, CD11b, L-selectin, CD44, and CD16 expression used to determine if functional AR were expressed in each activated PMN subpopulation and to determine if biological differences were apparent when comparing between PMN subpopulations.
2. No significant change in expression is shown as "-".
3. Significant decreases in expression are presented as red "↓"
4. Significant increases in expression are presented as green "↑"

6.0 DISCUSSION

A systematic survey of AR gene expression in blood leukocytes has not been completed in any species. This knowledge gap limits our understanding of AR subtype expression when comparing leukocyte populations within or among species. Previous research has demonstrated antibodies generated for ARs frequently lack specificity and are not an effective tool to discriminate among individual ARs. For example, antibodies reported to be specific for individual α1-AR subtypes gave a false positive reaction in mice following knockout of the relevant α1-AR subtype (Jensen, Swigart and Simpson, 2009). It has also been observed that antibodies targeting G-protein coupled receptors tend to lack specificity for these receptors. Although the reasons for this non-specificity remain unclear, it is theorized the non-specificity may, in part, be due to the similarity of all proteins in the G-protein coupled receptor family (Michel, Wieland and Tsujimoto, 2009). Furthermore, ligand binding studies (see section on Adrenergic Agonist and Antagonist Specificity) have also been limited by a lack of well-defined specificity for individual AR subtypes. Although transcript abundance does not always correlate directly with protein expression, highly specific methods such as RT-qPCR can provide more detailed information regarding probable expression of individual AR subtypes (ex: α2A vs. α2C-ARs) within specific leukocyte subpopulations. Quantifying transcript for individual AR genes provides possible insight into why specific leukocyte subpopulations may differ in their responses to stress or treatment with adrenergic agonists or antagonists. In this investigation, AR gene expression was first analyzed in bovine total blood leukocytes to determine if all 9 AR genes were expressed and to determine if AR gene expression was altered by stress. Cell isolation methods were then used to determine if AR gene expression varied significantly among

leukocyte lineages and subpopulations within lineages. These analyses provided a systematic survey of all 9 AR genes expressed in bovine blood leukocytes.

Leukocytes isolated from whole blood expressed the α1A- and α2A-AR genes at significantly higher levels than all other AR genes, suggesting these two ARs may play important roles in regulating bovine leukocyte function. Analysis of AR transcript in total blood leukocytes isolated from suckling calves, however, identified 4 AR genes that were significantly up-regulated following maternal separation (weaning) and transportation. These significant changes in AR gene expression involved the α2A- and all three β-ARs suggesting not only α- but also β-ARs may play important roles in SAM axis mediated effects on bovine leukocyte function. Changes in AR gene expression following stress suggested that either AR gene expression was altered in blood leukocytes or stress induced changes in the cellular composition of blood leukocytes, known as a stress leukon. Changes in the cellular composition of circulating blood leukocytes may have contributed to the altered transcript abundance observed for 4 AR genes.

Therefore, I addressed the question whether AR gene expression varied significantly among leukocyte subpopulations by separating blood leukocytes into individual cell lineages. Comparisons among leukocyte lineages revealed significant differences in AR gene expression. For example, there was the novel observation that the α2A-AR was expressed at a significantly higher level in B cells than all other leukocyte lineages analyzed. Furthermore, β2-AR gene expression was highest in the ILC subpopulation, consistent with previous reports regarding β2-AR protein expression by human NK cells (Jetschmann *et al.*, 1997). These observations not only confirmed bovine leukocyte lineages differed substantially in the expression of individual AR genes, but also suggested that individual ARs may serve different functions within each

leukocyte lineage. Furthermore, these observations suggested there may be similarities in leukocyte AR expression when comparing among mammalian species, such as bovine and human leukocytes. Therefore, studying neuroimmune interactions in one mammalian species may have relevance for other species.

I then investigated whether functional AR were expressed by bovine leukocytes. The AR gene family is complex with three members within each of the α1-, α2-, and β-AR subtypes. To reduce the complexity of this analysis, I focused on a single leukocyte lineage for which sufficient cells could be isolated to perform *in vitro* function studies. PMNs were chosen for their availability in blood and their important role in inflammation and host responses to a wide variety of infectious diseases. In particular, PMNs play an important role in the pathogenesis of bovine respiratory disease (McGuire and Babiuk, 1984; Slocombe *et al.*, 1985; Breider *et al.*, 1988; Caswell *et al.*, 1998; Li *et al.*, 2002) and stress has also been implicated as an important factor in this disease complex (Taylor *et al.*, 2010; Hodgson *et al.*, 2012). Therefore, understanding the response of PMNs to adrenergic agonists may provide further insight into mechanisms by which stress increases the risk of fatal bovine respiratory disease (Hodgson et al. 2012). To achieve my objectives, I first developed methods to work with both resting and activated PMNs and to analyze separately the responses of neutrophils and eosinophils to a variety of adrenergic agonists.

Previous investigations of adrenergic receptors and adrenergic agonists usually analyzed PMNs collectively (Haskó *et al.*, 1995a; Gu and Seidel, 1996; Szelenyi *et al.*, 2006; Trabold, Gruber and Fröhlich, 2007; LaBranche, Ehrich and Eyre, 2010; Scanzano *et al.*, 2015). There are no previous studies that directly compared AR gene expression and responses to adrenergic agonists in bovine neutrophils and eosinophils. There is also no prior information on bovine

eosinophil AR gene expression and responses to adrenergic agonists. Furthermore, there is no published evidence for either the expression or function of α-ARs in human eosinophils. Eosinophils can comprise almost a third of bovine blood PMNs depending on the individual animal and the time of year (Figure 5.14). My studies also determined that bovine neutrophils and eosinophils differ significantly in the expression of critical adhesion molecules (Figure 5.15), responses to activation stimuli (Figure 5.16), expression of AR genes (Figure 5.8), and responses to adrenergic agonists. Thus, these studies highlight the importance of analyzing individual PMN subpopulations when asking questions regarding the potential role of this leukocyte lineage when immune responses are altered by stress.

The expression of functional ARs by bovine neutrophils and eosinophils was confirmed using the endogenous agonists E and NE and synthetic agonists selected for known specificity for individual AR families. All adrenergic agonists studied had an effect on both resting and activated neutrophils and eosinophils. The greatest effect by adrenergic agonists was observed when analyzing iROS expression (Figures 5.17 to 5.20) and the expression of the adhesion molecules CD11b (Figures 5.21 to 5.24) and L-selectin (Figures 5.25 to 5.28). Collectively these studies provided evidence all three AR families are functional on bovine neutrophils and eosinophils. Furthermore, contradictory responses were observed when neutrophils were stimulated with synthetic adrenergic agonists targeting an individual AR family versus the endogenous agonists, E and NE. Thus, it was not possible to identify a single AR family that was the primary mediator of PMN responses to endogenous adrenergic stress hormones. Another possibility is that E and NE bind multiple ARs and PMN responses to these endogenous agonists reflects the combined signaling of multiple receptors within and possibly among AR families.

6.1 Adrenergic Receptor Gene Expression in Bovine Leukocytes

6.1.1 Adrenergic Receptor Gene Expression in Whole Blood Leukocytes

Transcript was detected for all 9 AR genes when RNA was isolated from leukocytes present in bovine blood (Figure 5.1). Expression of the α1A- and α2A-AR genes was significantly higher than all other AR genes, suggesting these two α-ARs may play predominant roles in mediating stress responses in bovine leukocytes. For the β-ARs, a transcript abundance ranking of β2 > β1 ~ β3 was observed, with both β1-ARs and β3-ARs exhibiting low relative expression that was near the limit of detection. In comparison, Mei et al. (2018), measured β-AR transcript in bovine spleen, among other tissues. They observed a transcript abundance ranking of β2 > β1 while β3-AR transcript was nearly undetectable. Thus, similar expression was observed in bovine spleen and leukocytes isolated from whole blood. Gene expression studies for all 9 ARs have not been previously published for leukocytes isolated from whole blood of any species that I am aware. However, the expression of some AR genes had been analyzed in human PBMCs and PMNs.

6.1.2 Effect of Stress on Adrenergic Receptor Gene Expression in Bovine Leukocytes

An investigation of AR gene expression in bovine blood leukocytes was completed following maternal separation (weaning) and transportation of suckling beef calves (Malmuthuge et al. Manuscript submitted). Weaning of suckling beef calves causes a transient increase in cortisol and a more sustained increase in NE concentrations (Hickey et al., 2003). In another study, weaning increased both E and NE concentrations in beef calves (Lefcourt and Elsasser, 1995). Transportation stress has previously been reported to increase blood cortisol concentrations (Buckham Sporer *et al.*, 2008; Ishizaki and Kariya, 2010) and NE concentrations

(Odore *et al.*, 2004). Thus, it is well established that weaning and transportation can each induce a SAM response with elevated levels of both E and NE.

A number of changes in AR gene expression were observed in response to weaning and transportation in calves. Our analysis demonstrated a significant up-regulation of β2-AR transcript at early time points (Days 2 and 4) following weaning and weaning + transportation (Figures 5.3 and 5.4). There was also a significant upregulation of α2A-AR transcript on Day 4 in the weaning group when compared to suckling calves. In addition, there was a much later (Day 28 post-weaning) up-regulation of the β1-AR and β3-AR genes in calves subjected to weaning + transportation. No such AR response was observed in the group exposed to just the weaning stressor alone, indicating the two types of stressors may elicit different immune responses. Interestingly, no previous study has investigated changes in the immune system 28 days following weaning and transportation. This data implies a combined stressor of weaning and transportation may have both short and long-term effects on the capacity of the bovine immune system to respond to adrenergic agonists.

Increased AR gene expression in bovine leukocytes following weaning and transportation may be the result of several causes. Catecholamines or β-AR agonists administered exogenously or generated *in vivo* by stress have previously been demonstrated to increase expression of β-ARs on human PBMCs (reviewed in Maisel et al., 1990). Glucocorticoids can also up-regulate the expression of AR genes (Hadcock, Wang and Malbon, 1989; Hadcock and Malbon, 1993). Increased activation of immune cells by inflammatory stimuli has also been associated with an up-regulation of AR gene expression (Rouppe Van Der Voort *et al.*, 2000a, 2000b; Scanzano *et al.*, 2015; Honke *et al.*, 2020). Thus, catecholamines, cortisol, or inflammatory stimuli may have contributed to the up-regulation in AR gene expression observed following weaning and

transportation stress. However, body temperatures were monitored weekly throughout the study and only three calves had transient increases in temperature above 39°C at a single time point and none of the calves were diagnosed with or treated for respiratory disease.

Up-regulation of AR gene expression following weaning and transportation suggests a possible increased capacity for neuromodulation of leukocyte functions. Some evidence for increased expression of AR genes correlating with an increased capacity for neuromodulation of leukocyte function has been reported. Scanzano et al. (2015) reported no effect by adrenergic drugs on resting human PMNs. However, following activation of human PMNs with fMLP, expression of 6 of the 9 ARs genes increased significantly. Furthermore, following activation with fMLP E, NE, isoproterenol, and other adrenergic drugs significantly altered PMN production of ROS, cell morphology, and expression of CD11b/CD18. Thus, activation by fMLP increased AR gene expression in human PMNs and the cells were more sensitive to adrenergic modulation of function. Similarly, increased expression of ARs on bovine leukocytes post-weaning and transportation may make them more sensitive to the effects of E and NE. Both E and NE had pro-inflammatory effects on bovine PMNs (Tables 5.2 and 5.3), suggesting increased expression of ARs by bovine leukocytes may increase pro-inflammatory responses if there is co-incidental activation of the SAM system.

Further research is needed, however, to determine if ARs also mediate pro-inflammatory responses by other leukocyte subpopulations. A major limitation of the suckling calf stress study was that all leukocyte lineages present in blood were analyzed together. Therefore, it is not known if β1, β2, β3 and α2A-AR gene expression was increased in one or more leukocyte lineages. Furthermore, stress can alter the cellular composition of blood leukocytes which is referred to as a stress leukon (Hickey, Drennan and Earley, 2003; Yagi *et al*., 2004; Ishizaki and

Kariya, 2010; O'Loughlin *et al*., 2012; Lynch *et al.* 2010). Therefore, another possibility is that altered AR gene expression in blood leukocytes following weaning and transportation may simply reflect changes in the cellular composition of leukocytes circulating in blood. Our RT-qPCR analysis of AR gene expression in individual leukocyte lineages (Figures 5.6 and 5.8) revealed transcript abundance for individual AR genes varied greatly among individual bovine leukocyte lineages. Therefore, future studies investigating the effect of stress on AR gene expression would be more informative if RNA was isolated from individual leukocyte lineages.

6.1.3 Adrenergic Receptor Gene Expression in Leukocyte Subpopulations

Bovine PBMCs and PMNs expressed detectable levels of transcript for all 9 AR genes (Figure 5.5). The β2 and α2-AR protein has been detected on human PBMCs (Blankesteijn *et al.*, 1993). Expression of the α2A, β2, and β1-AR genes has also been studied in human PBMCs (Light *et al.*, 2009; White *et al.*, 2012). A numerical difference was reported for transcript abundance of these three genes in PBMCs, although transcript abundance for individual AR genes was not statistically analyzed. Light et al. (2009) reported PBMCs isolated from control subjects expressed AR genes with transcript abundance ranking β2 > α2A > β1. White et al. (2012) reported a transcript abundance ranking of β2 > β1 > α2A. By comparison, ranking of AR gene transcript abundance in bovine PBMCs indicated α2A > β2 > β1. These differences in the ranking of β2-AR and α2A-AR transcript abundance when comparing human and bovine PBMCs suggests β2-AR may play a more prominent role in human PBMCs, whereas the α2A-AR may play a more important role in bovine PBMCs. Alternatively, these differences in AR gene expression may simply reflect differences in the cellular composition of bovine and human PBMCs that influences the overall transcript abundance of individual AR genes.

Interestingly, mRNA for α1A-, α1B-, and α1D-AR genes was not detected in PBMCs isolated from healthy humans (Rouppe van der Voort *et al.*, 1999). However, when PBMCs were treated with dexamethasone, a glucocorticoid receptor agonist, or phytohemagglutinin, a T-cell mitogen, then expression of all three α1-AR genes was detected. Furthermore, PBMCS isolated from patients with juvenile rheumatoid arthritis, an inflammatory disease, expressed all three α1-AR genes with the α1D-AR gene being the most abundant (Rouppe van der Voort *et al.*, 2000b). These ARs were also shown to be functional on PBMCs isolated from these patients (Heijnen *et al.*, 1996). In contrast, all six α-ARs were detected in PBMCs isolated from healthy cattle. These differences suggest that α-ARs play a more important role in bovine than human PBMCs.

Expression of all 9 AR genes has also been studied in human PMNs (Scanzano *et al.*, 2015). Transcript for the α2B-AR gene was not detected in human PMNs. In bovine PMNs, transcript for the α2B-AR gene was detectable at a very low level. In bovine PMNs, the ranking of AR gene transcript abundance indicated expression varied with α2A ~ β2 ~ α1A > α1B ~ β1 ~ β3 > α2C ~ α2B ~ α1D. Although Scanzano et al. (2015) did not statistically analyze expression of AR genes in human PMNs, numerical differences were apparent in the data presented. Human PMNs had an approximate ranking of AR gene expression with β3 > β2 > α1A > α2A > β1 > α1B > α1D > α2C. Transcript abundance was greatest for both the β2- and α1A-AR genes in human and bovine PMNs, suggesting there may be conserved roles for these ARs in regulating granulocyte function. However, transcript abundance for the β3-AR gene differed markedly in bovine and human PMNs, suggesting that there may also be species-specific aspects to AR gene expression and function.

Bovine PBMCs and PMNs, when not separated into subpopulations, had similar transcript abundance for all AR genes, except for the α2A-AR gene. There were, however,

marked differences in the expression of individual AR genes within each population. Furthermore, when PBMCs and PMNs were separated into subpopulations more pronounced differences in AR gene expression were apparent. For example, expression of the α2A-AR gene was significantly ($p < 0.01$) higher in $CD21^+$ B cells than all other leukocyte subpopulations analyzed. B cells may be the primary population contributing to the higher α2A-AR gene expression observed in PBMCs. There are no previous reports of α-AR gene expression in either bovine B cells or B cells of other species.

Some previous research has been completed to analyze α-AR protein expression and function in lymphocytes. McPherson and Summers (1982) reported [3H]-clonidine, an α2-AR agonist that also binds 5-HT1A receptors at a lower level (Newman-Tancredi et al., 1998), reversibly bound to lymphocytes isolated from guinea pig spleens. Titinchi and Clark (1984) also observed binding of clonidine and yohimbine, another α2-AR antagonist, to human PBMCs. These authors did note, however, that platelets also have a high level of α-ARs and platelets may have been present in their PBMC preparations. In my experiments, platelets should not be present since high-speed cell sorting was used to isolate bovine B cells. In 1991, Goin et al. isolated lymphocytes from patients with B cell leukemia (leukocytes from these patients were 75-94% lymphocytes). Monocytes were depleted from lymphocyte preparations using plastic adherence in culture. These B cell enriched lymphocytes were noted to express a high density of α2-ARs as measured with [3H]-yohimbine. The α1-AR antagonist, [3H]-prazosin, bound at a low level but [3H]-dihydroalprenolol, a β-AR antagonist, was also observed to have significant binding. Treatment with isoproterenol increased cAMP levels while clonidine decreased cAMP levels. These responses provided evidence that both β-ARs and α2-ARs were functional in these cells. Although these preparations likely contained primarily B cells, some contaminating T cells

and ILCs may have been present. Furthermore, these B cells were isolated from patients with B cell leukemia and may not represent normal B cells. Thus, prior evidence that the α2-AR gene is expressed in non-transformed B cells circulating in blood remains ambiguous. However, previous research has indicated that α-ARs may be present and functional on lymphocytes, and potentially on normal B cells.

There is additional evidence that α2-AR agonists can alter B cell function, Sanders and Munson (1985) treated mouse splenocytes with either methoxamine, an α1-AR agonist, phenylephrine, an α1-AR agonist, or clonidine, an α2-AR agonist. Methoxamine increased IgM antibody production by 63% 4 days after treatment. Phenylephrine treatment decreased IgM antibody production on day 5 but increased IgM antibody production by 50% on day 6 and 64% on day 7. In contrast, clonidine induced a 50% decrease in IgM antibody production five days after treatment. These results provide evidence that α1-ARs can increase antibody production but α2-ARs inhibited antibody production. However, a major limitation of these studies is that they did not use purified B cell populations. Thus, the possibility remains that the AR agonists had indirect effect on B cells by stimulating other cells present in the splenocyte preparation (Kohm and Sanders (2001). However, Honke et al. (2020), used magnetic activated cell sorting to isolate murine splenic B cells to 98% purity. Following CpG ODN treatment, the purified B cells were then treated with either the α1-AR agonist phenylephrine or the α2-AR agonist dexmedetomidine hydrochloride. Both AR agonists induced significant reductions in IL-10 production, providing direct evidence for α-AR activity on B regulatory cells.

There are no published reports of α-AR gene expression in B cells of any species. Furthermore, there is no direct evidence that either human or bovine B cells respond to either α1- or α2-AR agonists. Analysis of AR transcript (Figure 5.6) does, however, indicate α2A-AR is

expressed at a high level by $CD21^+$ B cells present in bovine blood. Transcript for α2B- and α2C-ARs was either undetectable or just at the level of detection. Functional α1-ARs may also be expressed by bovine B cells since transcript was detectable for both α1A- and α1B-ARs but α1D-AR gene transcript was very low. Further analysis of both α-AR expression and function in bovine B cells may provide insight into how stress and certain drugs, such as the anesthetic dexmedetomidine or xylazine, may impact antibody responses.

Significantly higher β2-AR gene expression was also observed in bovine ILCs, monocytes, and eosinophils relative to other blood leukocyte populations (Figure 5.6 and 5.7). High relative expression of β2-AR protein (1420 sites/cell) has been reported for human NK cells. Similar to my results for transcript expression, a lower density of α1-ARs (663 sites/cell) and α2-ARs (397 sites/cell) was also observed on NK cells (Jetschmann *et al.*, 1997). High expression of the β2-AR gene in ILCs and monocytes is also consistent with previous studies of β-AR protein expression in PBMCs isolated from healthy humans. In human PBMC subpopulations, the relative abundance of β-ARs was reported to be NK cells > monocytes ~ B cells ~T suppressor/cytotoxic > T helper cells (Maisel *et al.*, 1990). A similar relative abundance for β2-AR gene transcript in bovine PBMC subpopulations was observed with ILCs > monocytes > T cells ~ B cells. This suggests that the expression and possibly the function of the β2-AR may be similar in bovine and human ILCs and cattle may provide a good model for studying the effects of stress and adrenergic agonists or antagonists on ILC function.

T cells were also observed to express α2A-AR mRNA, albeit at a significantly lower level relative to that observed in B cells and ILCs. Transcript for other α-ARs was also detected in T cells. Thus, when splenocytes or lymphocytes were tested in other species, these cells may have contributed to the binding of radiolabeled α-AR ligands. It is important to note that bovine

T cells were not fractionated into specific subpopulations, including CD4+ T helper lymphocytes, CD8+ cytotoxic T lymphocytes, and T regulatory cells. Thus, expression of AR mRNA may vary substantially among specific bovine T cell subpopulations, as has been demonstrated in other species (Maisel *et al.*, 1990).

When PMNs were fractionated, neutrophils expressed a higher level of most ARs relative to eosinophils (Figure 5.7). However, the α2A- and β2-AR genes were expressed at significantly higher levels in eosinophils relative to neutrophils. Previous research demonstrated both the expression and function of β2-ARs by human, mouse, and guinea pig eosinophils (Yukawa *et al.*, 1990; Noguchi *et al.*, 2015; Kainuma *et al.*, 2017). Thus, the current result for β-AR gene expression in bovine eosinophils is consistent with results from a number of other species.

No research has quantified the expression or reported evidence for functional α-ARs in either human or bovine eosinophils. Expression of the α1A-AR gene was observed with bovine eosinophils isolated from blood while transcript for both α1B and α1D-AR genes was barely detectable. There was also robust expression of the α2A-AR gene with transcript barely detectable for both the α2B- and α2C-AR genes (Figure 5.7). My studies also provided the first evidence that both the α1- and α2-ARs were functional, mediating eosinophil responses to specific agonists (Tables 5.2 and 5.3). These data suggest that the role α-ARs play in regulating bovine and human eosinophils responses should be studied further.

It should be noted, however, that the expression of ARs by eosinophils may depend on the immune compartment or tissue from which these cells are isolated. Liu *et al.* (2020) analyzed the expression of all 9 ARs genes in eosinophils isolated from the murine conjunctiva. Expression of the α1A-AR gene was detected but there was not a detectable level of transcript for the remaining 8 AR genes. Thus, eosinophils localized to specific tissue sites may change AR

gene expression, indicating transcript analyses with eosinophils isolated from blood should not be extrapolated to eosinophils present in other tissues. It may be of considerable value to compare AR gene expression and function on the same leukocyte subpopulations isolated from blood and specific tissue locations, such as the lung or gut. This may reveal whether the response of a leukocyte subpopulation during a stress response can vary at different sites throughout the body.

High expression of the β2-AR gene was also observed in bovine monocytes (Figure 5.6). This is consistent with a previous study of β-ARs on human monocytes (Maisel *et al.*, 1990). Furthermore, although macrophages are the tissue-differentiated form of monocytes, this result is consistent with a previous study that demonstrated β2-ARs were functional on bovine alveolar macrophages (Gu and Seidel, 1996). Similar to B cells, undetectable (>35 PCR cycles) levels of α1D- and α2B-AR transcript were observed in bovine monocytes and they also expressed very low levels of α2C-AR gene transcript. Thus, it is unlikely that there is substantial protein expression or function associated with these AR subtypes. However, adrenergic agonist studies with bovine monocytes and macrophages would be required to determine if AR signaling was restricted primarily to the β2-AR.

The three most highly expressed AR genes among the bovine leukocyte subpopulations analyzed included β2-, α2A-, and α1A-ARs. These three AR genes were also associated with significant differences in transcript abundance when comparing among leukocyte subpopulations. Further study of individual AR subtypes may reveal unique differences in the functional responses mediated by these receptors when comparing among bovine leukocyte lineages. Variation in AR gene expression when comparing among leukocyte subpopulations reveals substantial potential for individual leukocyte lineages to respond differently to either

stress-induced release of catecholamines or the therapeutic administration of synthetic adrenergic agonists or antagonists. This lineage-specific variation in leukocyte AR gene expression may provide insight into the many contradictory reports that stress can either enhance or inhibit immune responses.

6.2 Bovine Neutrophils and Eosinophils

PMNs were selected for further analysis of AR gene expression and AR function since neutrophils play an important role in inflammation and mediate much of the lung pathology observed during BRD (McGuire and Babiuk, 1984; Slocombe *et al.*, 1985; Breider *et al.*, 1988; Caswell *et al.*, 1998; Li *et al.*, 2002). Also, it was hypothesized that modulation of neutrophil function by E and NE may be one mechanism by which stress increases BRD mortality. Furthermore, PMNs are easily separated from blood with minimum handling or manipulation and culture conditions have been optimized for the *in vitro* study of bovine PMNs (Whale *et al.*, 2006). Agonists specifically targeting the β2-AR were previously shown to alter bovine PMN responses (LaBranche, Ehrich and Eyre, 2010) and there was evidence from Holroyde and Eyre (1976) that α1-ARs inhibited histamine release by bovine PMNs stimulated with a high concentration (10^{-3} M) of E. When Diez-Fraile et al. (2000) tested the α1-AR agonist phenylephrine with whole blood leukocytes, they reported no effect on bovine PMN expression of CD11b. However, this non-responsiveness may have been due to indirect effects by other cells in the culture, such as T cells, B cells or monocytes. There have been no studies of the direct effects of α2-AR agonists on bovine PMNs. However, Amouzadeh (1991) reported high concentrations of xylazine, an α2-AR agonist used as a veterinary sedative, caused death in cattle by pleural effusion. Some evidence was provided that this treatment may have induced TNFα and ROS mediated damage in the lung but this pathology was not directly linked to PMNs. In

contrast, α2-ARs have been found to mediate primarily anti-inflammatory responses in humans following administration of the anaesthetic dexmedetomidine, an α2-AR agonist. These responses included decreased levels of TNFα, IL-6 and C-reactive protein (Kallioinen *et al.*, 2019; Wang *et al.*, 2019b). My research provides a systematic analysis of AR gene expression and AR function in bovine neutrophils and eosinophils which may be useful for better understanding interactions between stress, inflammation, and infectious disease in cattle.

6.2.1 Optimization of PMN Culture and Activation Conditions

PMN activation conditions were optimized for the recovery of viable single cell suspensions to facilitate flow cytometric analysis of iROS and protein expression by individual cells. Flow cytometric analyses provided a quantitative measure of responses induced by adrenergic agonists and facilitated an independent analysis of neutrophil and eosinophil responses. Several new criteria for measuring the activation of bovine neutrophils and eosinophils were also optimized. Quantitative measures of neutrophil and eosinophil activation included an analysis of iROS and the expression of CD16, L-selectin, CD44, and CD11b. These parameters revealed how adrenergic agonists may alter the activation state of both resting and activated bovine neutrophils and eosinophils.

Culture media was previously optimized to support PMN survival in culture for three days (Whale *et al.*, 2006) which facilitated an analysis of both neutrophil and eosinophil responses with minimal loss of viable cells. Published information was used initially to identify a dose of BoZ that induced bovine PMN activation. Stimulation of bovine PMNs with 1 mg BoZ/mL was reported to activate a variety of bovine PMN functions (Brown and Roth, 1991) but this protocol was not used for flow cytometric analyses or long term assays of cell function. We confirmed BoZ induced a dose-dependent increase in bovine PMN iROS activity (Figure 5.10)

but there was also a dose-dependent increase in apoptosis and cell death, resulting in decreased recovery of viable cells (Figure 5.9). Treatment of PMNs with as little as 0.05 mg BoZ/mL resulted in BoZ particle uptake by neutrophils and eosinophil degranulation (Figure 5.11A). However, cell clumping was still observed with this much lower dose of BoZ, which was also evident in flow cytometry as increased FSC and SSC (Figure 5.12). As a result of these observations, the use of BoZ as an activating agent for bovine PMNs was further optimized to ensure recovery of a viable, single cell suspension of bovine PMNs.

Several different PMN activation protocols were evaluated to identify stimuli that would provide viable, single cell suspensions which would be compatible with flow cytometric analyses. Furthermore, PMN culture temperature was changed from 37°C to 39°C, to better represent normal bovine body temperature (Wrenn, Bitman and Sykes, 1958). The final PMN activation protocol used for my research combined a much lower dose of BoZ (6.25 μg/mL) than used in previous studies (Sample and Czuprynski, 1990; Brown and Roth, 1991) and co-stimulation with 10 ng/mL rBoIFNγ. PMNs recovered following this co-stimulation were > 99% viable and > 85% of the input cells were recovered (Figure 5.9). Also, the recovered PMNs did not clump, as confirmed by cytospins (Figure 5.11B) and flow cytometric profiles of FSC and SSC (Figure 5.13). PMN activation following co-stimulation with BoZ and rBoIFNγ was evident with ingestion of BoZ in neutrophils (Figure 4.6 and 5.11) and eosinophils (Figure 4.6 and 5.11). Furthermore, both neutrophils and eosinophils displayed a broad activation response to this combined stimulus (Figure 5.16). Finally, a flow cytometric analysis gate was set based on FSC and SSC that excluded doublets (Figure 5.13) and ensured changes in the expression of iROS and surface proteins reflected the responses of individual cells.

6.2.2 Percentage Neutrophils and Eosinophils in PMN Preparations

There are few well-established methods to separate neutrophil and eosinophil populations within bovine blood PMNs. Separation of bovine eosinophils was previously described based on the use of Percoll density gradients (Freiburghaus and Jörg, 1990). However, this method took a significant amount of time and required large volumes of blood (i.e 6 L). Therefore, I developed a new method to discriminate between neutrophil and eosinophil subpopulations in PMNs isolated from blood. Following multicolour staining of PMN surface proteins, a distinct subpopulation of CD44 low, L-selectin high, FL-1 high cells was identified (Figure 5.15, 5.7, 4.8 and 4.9). This subpopulation had an abundance similar to the frequency of eosinophils observed in isolated bovine PMN preparations. High-speed cell sorting confirmed this subpopulation was indeed eosinophils (Figure 4.5 and 4.6) and the CD44 high subpopulation was neutrophils. Previously, high-speed sorting was used to isolate bovine blood eosinophils based on their higher autofluorescence in FL-1 relative to neutrophils (Anderson et al. Manuscript in preparation). High-speed cell sorting to separate neutrophils and eosinophils for RT-qPCR analysis of AR gene expression in the present project replicated these previous results. Furthermore, the high autofluorescence subpopulation in both FL-2 and FL-3 channels was also confirmed to be eosinophils and the autofluorescence low subpopulation was confirmed to be neutrophils. These methods were effective with both resting and BoZ + rBoIFNγ co-stimulated cells (Figure 4.4). High-speed sorting was used to validate the flow cytometry gates used to discriminate between neutrophils and eosinophils in bovine PMNs, facilitating a separate analysis of these two subpopulations following stimulation with adrenergic agonists.

Diez-Fraile et al. (2003) reported identifying neutrophils and eosinophils as distinct subpopulations in whole blood leukocytes based on differences in cell size (FSC) and complexity

(SSC). Eosinophils were identified as a cell population with much higher SSC and lower FSC than neutrophils. However, the identity of the alleged eosinophil population was never independently confirmed and when we performed high-speed sorting of neutrophils and eosinophils there was very little difference in the FSC and SSC profiles of the purified subpopulations.

Diez-Fraile et al. (2003) also reported an intracellular staining method to identifying bovine neutrophils and eosinophils using three mAbs, CH138A, Du12-116b and VPM65. Intracellular staining required fixation and permeabilization of cells, and again no direct evidence was provided to confirm this antibody staining protocol effectively discriminated between neutrophils and eosinophils. This paper also described L-selectin and CD11b/CD18 on bovine neutrophils and eosinophils. However, without validation of the specificity of neutrophil and eosinophil identification, the validity of this data is questionable.

The percentage eosinophils in PMNs isolated from bovine blood varied substantially among individual animals and with time of year (Figure 5.14). A significantly higher percentage of eosinophils was present in PMNs isolated from cattle during late summer and autumn than during spring in Saskatoon (SK, Canada). Thus, the abundance of eosinophils in isolated PMNs could significantly alter responses measured to a specific stimulus when neutrophils and eosinophils differ in their response to this stimulus. Historically, the potential impact of eosinophils on PMN responses was largely ignored. For example, LaBranche, Ehrich and Eyre (2010) studied bovine PMNs, as opposed to analyzing neutrophils and eosinophils independently when measuring responses induced by the β2-AR. Furthermore, one of the most recent and detailed studies characterizing adrenergic function in human neutrophils actually studied PMNs as opposed to purified neutrophils (Scanzano *et al.*, 2015). Therefore, in my studies I developed

methods to separately analyze neutrophil and eosinophil responses when working with bovine PMNs.

6.2.3 Basal Expression of Activation Markers on Resting Neutrophils and Eosinophils

Notable similarities and differences were observed when comparing the five parameters used to detect neutrophil and eosinophil activation. CD16 was nearly undetectable (< 1.0% positive cells) on resting eosinophils but a low level of CD16 was observed on a larger subset of neutrophils (< 30.0% positive cells). Human eosinophils are reported to lack CD16 expression, except in individuals with inflammatory or allergic conditions (Davoine *et al.*, 2002, Davoine *et al.*, 2004). Detectable expression of CD16 on bovine neutrophils means this low-affinity Fc receptor may be involved in mediating responses such as NETosis, as high CD16 expression has been associated with NET formation on human neutrophils (Millrud *et al*., 2017) or phagocytosis (Huizinga *et al.*, 1990; Kimberly *et al.*, 1990).

No significant difference was observed when comparing basal expression of iROS and CD11b by resting bovine neutrophils or eosinophils (Figure 5.15). This observation supports the conclusion that neutrophils and eosinophils circulating in blood have similar iROS metabolism and possibly CD11b-mediated adhesion to vasculature (Coxon *et al*., 1996; Ince, Weber and Scheiermann, 2019). Furthermore, these two parameters couldn't be used to differentiate between neutrophils and eosinophils. In contrast, L-selectin and CD44 expression were markedly different when comparing resting neutrophils and eosinophils. This is the first time CD44 expression has been analyzed on bovine eosinophils but CD44 expression was previously analyzed on bovine neutrophils (Gonen *et al*., 2008; Blagitz *et al*., 2017). The specificity of lower CD44 expression on eosinophils was confirmed with high-speed cell sorting (Figure 4.5 and 4.6) and CD44 provided an excellent marker to differentiate between both resting and

activated neutrophils and eosinophils. Differential L-selectin expression could also be used to separate neutrophils from eosinophils (data not shown) but high-speed sorting of L-selectin^{Hi} and L-selectin^{Lo} PMN subpopulations revealed there was not complete separation of neutrophils from eosinophils based on this parameter alone.

CD44 and L-selectin are important molecules involved in regulating cell migration, and differential expression of these molecules by bovine neutrophils and eosinophils suggests the migration and retention of these two populations in tissues may also differ. L-selectin plays an important role in cell adhesion to endothelium, a first step in cell homing (Ivetic, Green and Hart, 2019). Higher L-selectin expression by eosinophils circulating in bovine blood may augment greater eosinophil adhesion and rolling on vasculature relative to neutrophils. CD44 binds to hyaluronan in the extracellular matrix and this interaction can slow cell migration through tissues (Wang *et al*., 2002). Higher CD44 expression by neutrophils may impede their migration through tissues when compared to eosinophils. Thus, modulation of CD44 and L-selectin expression by catecholamines may have important effects on neutrophil and eosinophil migration and localization at sites of inflammation during stress responses.

6.2.4 Neutrophil and Eosinophil Activation Responses

Co-stimulation of bovine PMNs with rBoIFNγ and BoZ increased both iROS and CD11b expression (Figure 5.16). Increased iROS and CD11b expression has previously been reported for activated bovine PMNs (Sample and Czuprynski, 1990; Brown and Roth, 1991; Diez-Fraile *et al.*, 2000). Sample and Czuprynski (1990) reported a synergistic effect on ROS production in bovine PMNs following co-stimulation with rBoIFNγ and BoZ. They measured ROS using several different methods. Luminol dependent chemiluminescence was used to measure primarily O_2- superoxide anions that are both intracellular and extracellular (Bedouhène et al.,

2017). They also measured superoxide anions with a cytochrome C oxidase assay and hydrogen peroxide production with a colorimetric microassay. My flow cytometric assay used DCFDA to indirectly measure a variety of secondary intracellular ROS, including alkoxyl, peroxyl, $CO_3\cdot$(carbonate) and OH· oxygen radicals (Halliwell and Whiteman, 2004). Sample and Czuprynski (1980) reported an approximately 40% increase in ROS activity with their activation protocol and subsequent ROS measurement assay. However, Sample and Czuprysnki (1980) used a much larger dose of BoZ (~1 mg/mL). With a much lower dose of BoZ (6.25 μg/mL) and rBoIFNγ co-stimulation there was a 58% increase in neutrophil iROS and a 65.4% increase in eosinophil iROS (Figure 5.16; Table 5.1 and 5.2). Thus, my PMN stimulation protocol and iROS detection system was at least as effective as that published previously which used over a 150-fold higher dose of BoZ.

Neutrophil activation studies have been performed with cells isolated from multiple species. Human neutrophils have previously been shown to "shed" L-selectin in response to activating factors such as Toll-like receptor (TLR) agonists (Hayashi, Means and Luster, 2003) and phorbol myristate acetate (PMA) (Li et al., 2006). A variety of activating factors have also been found to have differing effects on L-selectin "shedding" by bovine PMNs. Diez-Fraile et al. (2003) reported that when bovine whole blood samples were incubated with platelet activating factor (PAF) for one hour at 37°C then decreased L-selectin was detected on PMNs, identified by FSC and SSC during a flow cytometric analysis. However, Swain et al. (1998) reported L-selectin levels increased on bovine PMNs when whole blood samples were incubated for 15 minutes at 37°C with PAF. L-selectin expression in this study was also analyzed with flow cytometry using FSC and SSC to identify PMNs. The difference in PMN responses to PAF in these two studies may have been due to differences in the post-stimulation time when L-selectin

expression was analyzed or the use of different diluents to dissolve the PAF. In my studies, I observed that both bovine neutrophils and eosinophils "shed" L-selectin in response to a combined stimulus of BoZ + rBoIFNγ, and that eosinophils shed L-selectin at a significantly greater level than neutrophils in response to this stimulation (Figure 5.16; Table 5.1 and 5.2).

CD44 expression by human neutrophils has also been reported to decrease in response to activating factors such as TNFα, fMLP and PMA (Campanero *et al.*, 1991). Activated T cells isolated from cattle infected with *Mycobacterium bovis* were reported to have reduced levels of CD44 (Waters *et al.*, 2003) but the effect of activating factors on CD44 expression by cultured bovine neutrophils and eosinophils has not been studied. In response to BoZ + rBoIFNγ, bovine neutrophils were observed to significantly decrease their expression of CD44, whereas eosinophils expressed no change in CD44 (Figure 5.16; Table 5.1 and 5.2).

The effect of co-stimulation with opsonized zymosan and IFNγ has also been studied in human neutrophils (Kowanko and Ferrante, 1987). This co-stimulation increased neutrophil production of hydrogen peroxide (H_2O_2), a form of ROS, as well as chemiluminescence. Chaves et al. (1996), confirmed opsonized zymosan and IFNγ co-stimulation of human neutrophils had a similar effect on ROS production. Thus, while the effect of opsonized zymosan and IFNγ co-stimulation on ROS production has been studied with PMNs from cattle and other species there has been no analysis of how this co-stimulation affects the expression of other proteins by neutrophils. This is the first report of CD11b up-regulation, L-selectin "shedding" and CD44 "shedding" on neutrophils following co-stimulation with opsonized zymosan and IFNγ (Figure 5.16; Table 5.1 and 5.2). However, the changes observed in these activation markers are consistent with previous studies that analyzed responses in activated neutrophils (Kowanko and

Ferrante, 1987; Sample and Czuprynski, 1990; Campanero *et al*., 1991; Diez-Fraile *et al*., 2000; Hayashi, Means and Luster, 2003; Li *et al*., 2006).

Studies have also been completed to analyze activation of human eosinophils. Human eosinophils "shed" L-selectin and up-regulated expression of the CD11b/CD18 complex Mac-1 within 30 minutes after IL-5 and GM-CSF stimulation. However, it has also been shown that human eosinophils up-regulate L-selectin expression 12 hours after incubation with IFNγ (Momose *et al.*, 1999). The effect of opsonized zymosan on human eosinophils has also been studied and found to increase release of superoxide anions and eosinophil peroxidase (Yukawa *et al.*, 1990). However, the effect of opsonized zymosan and IFNγ co-stimulation has not been studied with eosinophils isolated from any species. The current investigation is the first report that this co-stimulation activates a broad range of responses in bovine eosinophils, inducing significant increases in iROS and CD11b expression and L-selectin "shedding".

Separate analyses of eosinophils and neutrophils revealed that decreased CD44 expression was unique to neutrophils and CD44 expression remained unchanged on activated eosinophils (Figure 5.16). Furthermore, significant differences were also observed when comparing expression of CD11b and L-selectin on neutrophils and eosinophils following co-stimulation with BoZ + rBoIFNγ (Figure 5.16). Thus, separate phenotypic analysis of activated bovine neutrophils and eosinophils confirmed that these two PMN subpopulations can differ in their responses in multiple ways. This further highlights the importance of not analyzing bovine PMNs as a single population when studying responses to stimuli that may alter cell function.

6.3 Adrenergic Receptor Function in Bovine Neutrophils and Eosinophils

The effects of the physiological agonists, E and NE, and synthetic agonists targeting α1- α2- and β-ARs were tested on both resting and activated bovine PMNs. Furthermore, the responses of neutrophils and eosinophils were analyzed separately. This discrimination between the two major PMN subpopulations revealed for the first time that neutrophils and eosinophils not only differ in their expression of AR genes but also display differences in their response to adrenergic agonists.

All adrenergic agonists evaluated had effects on both neutrophils and eosinophils. This not only provided evidence that both PMN subpopulations expressed functional ARs but also provided evidence that at least one member of each of the three AR families was able to transduce signals in neutrophils and eosinophils that altered cell function. AR signaling resulted in diverse cellular responses including regulating iROS activity and changing the expression of adhesion molecules, such as CD11b and L-selectin. These AR agonists also had selective effects on the expression of CD16 and CD44 in neutrophils and eosinophils.

Resting neutrophils responded to E and NE stimulation with a consistent pro-inflammatory response, including increased iROS production (Figure 5.17). BoZ + rBoIFNγ activated neutrophils demonstrated a similar response to NE but not to E. This pro-inflammatory effect was somewhat unexpected, as previous literature on E, NE, β2-AR agonists, and α2A-AR agonists reported a suppressive effect on activated human neutrophils (Trabold, Gruber and Fröhlich, 2007; Scanzano and Cosentino, 2015; Chen *et al*., 2016; Margaryan *et al*., 2017). In BoZ activated bovine PMNs, a suppressive effect on ROS production was reported with a β2-AR agonist (LaBranche, Ehrich and Eyre, 2010). Margaryan et al. (2017) similarly observed a suppressive effect by E with LPS-activated human PMNs. However, increased expression of

CD11b and IL-8 production was observed in resting human PMNs following stimulation with E, indicating a pro-inflammatory effect. This increase in CD11b is consistent with my results on resting bovine neutrophils and eosinophils. My research suggests that stress-induced release of catecholamines such as NE may further exacerbate inflammatory responses mediated by neutrophils circulating in blood and possibly by activated neutrophils present in either blood or tissues. The CD11b molecule has several functions in PMNs including being a complement receptor (complement receptor three; CR3) (Berends *et al*., 1993), influencing Fc-receptor mediated degranulation (Tang et al. 1997), and mediating adhesion to endothelium via ICAM-1 (Coxon *et al*., 1996; Ince, Weber and Scheiermann, 2019b). CD11b enhances PMN attachment to endothelium following the initial attachment and rolling on endothelium mediated by molecules such as L-selectin (Ivetic, Green and Hart, 2019). These results provide insight into a possible mechanism by which stress may further increase neutrophil activation while circulating in blood and also alter PMN migration.

O'Loughlin *et al*. (2011) reported weaning stress in calves induced a stress leukon characterized by a neutrophilia and increased expression of pro-inflammatory cytokine genes, and TLR4 in bovine leukocytes isolated from whole blood. Increased expression of TNFα, IFNγ, IL-8 and IL-1β genes was also observed and it was concluded that weaning stress enhanced rather than suppressed immune responses. In my studies, *ex vivo* stimulation of PMNs with E and NE similarly induced a pro-inflammatory response in bovine neutrophils and eosinophils. Thus, the *in vivo* inflammatory responses observed during weaning stress are consistent with activation of neutrophils and eosinophils by E and NE and further research is warranted to focus specifically on the expression and function of ARs in bovine neutrophils following maternal separation (weaning) and transportation.

Lynch et al. (2010) also reported decreased L-selectin expression on bovine blood neutrophils in weaned versus control animals. This effect was observed in conjunction with the classic short-term neutrophilia frequently observed during stress. In my experiments, decreased L-selectin expression was observed in response to adrenergic agonist stimulation of both resting (Figures 5.25) and activated (Figures 5.26) neutrophils. Thus, one possible mechanism by which increased L-selectin shedding occurred on blood neutrophils following weaning stress may be through increased stimulation by either E or NE. L-selectin attaches to glycans such as the sialyl Lewis^X (sLe^x) antigen on endothelium and initiates the first attachment and subsequent "rolling" of leukocytes, such as neutrophils, along the endothelium (Ivetic, Green and Hart, 2019). Thus, L-selectin shedding should reduce this first step of attachment and rolling. Lynch et al. (2010) did not study CD11b expression on bovine neutrophils, but there is evidence that L-selectin shedding reduces attachment and invasion of endothelium even if CD11b is elevated (Enders *et al.*, 1995). This may be one mechanism contributing to the short-term neutrophilia observed following stressors, such as weaning. For example, mice lacking ADAM17, the metalloprotease which cleaves L-selectin, in circulating neutrophils were compared with normal mice (Long *et al.*, 2012). Mouse neutrophils that could not shed L-selectin were found to have significantly increased invasion of inflamed tissues in response to E. coli infection, whereas mice that could shed L-selectin had greater numbers of circulating neutrophils (Long *et al.*, 2012). Short term exposure to stress hormones such as catecholamines in cattle appears to cause L-selectin shedding on neutrophils, possibly preventing egress of neutrophils from circulation and contributing to the classic neutrophilia which is part of the stress leukon. The neutrophilia observed as part of the stress leukon is usually attributed in part to a decrease in the marginal granulocyte pool (neutrophils transiently attached to endothelium), resulting in an increased

circulating granulocyte pool (Cornell University College of Veterinary Medicine, 2020). L-selectin "shedding" from marginated neutrophils may contribute to neutrophil detachment from endothelium and entry into the circulating pool. Thus, L-selectin shedding from neutrophils in response to catecholamines may be one mechanism by which short-term neutrophilia occurs in response to stress. Evidence for this theory exists, as catecholamine-induced neutrophilia is dominated by mature neutrophils in ruminants (Jones and Allison, 2007). It remains to be determined if this neutrophilia enhances or inhibits innate immune defenses during a stress response.

The current research also demonstrates a potentially important role for eosinophils during a stress response. Specifically, resting eosinophils increased CD11b, CD44, and CD16 levels in response to NE. Increased expression of these surface proteins has previously been identified as characteristic of an activated or allergic phenotype for eosinophils (In't Veen et al., 1998; Davoine *et al*., 2002; Katoh.*et al*., 2003) (Table 5.2). It is interesting to note that the level of L-selectin increased on resting eosinophils in response to NE. This increase in L-selectin expression would likely increase initial eosinophil binding and rolling on vasculature (Ivetic, Green and Hart, 2019), and firm adhesion by increased CD11b could also be promoted (Coxon *et al*., 1996; Ince, Weber and Scheiermann, 2019). Changes in L-selectin expression induced by NE, for example, suggest eosinophils circulating in blood may more readily migrate into tissues during a stress response and respond to pathogens or allergens present at these sites. In humans, NE injection was reported to decrease eosinophils in blood by 10% (Koch Weser, 1968) but similar studies have not been performed with cattle. Collectively, the *ex vivo* response of eosinophils to NE is indicative of increased cell activation with an increased capacity to migrate into tissues (Enders *et al.,* 1995). Understanding stress induced changes in eosinophil function

may help improve the control and treatment of conditions such as parasitic infections in cattle (Gånheim, Höglund and Waller, 2004; Doster, 2010; Shapiro, Peregrine and Caswell, 2017) and in other species such as pigs, or even humans. These changes in eosinophil function may also be important for understanding the pathogenesis of allergic reactions such as allergic rhinitis in cattle (Van Metre, 1997).

One surprising result was that the responses of neutrophils and eosinophils to synthetic AR agonists targeting individual AR families often differed from responses observed with E and NE. Increased CD11b expression and L-selectin "shedding," were generally consistent with granulocyte activation and these responses were observed with both E and NE and synthetic agonists targeting individual AR subtypes. However, synthetic agonists specific for individual ARs subtypes induced a significant decrease in iROS expression in both resting and, less consistently, in activated neutrophils and eosinophils (Figures 5.17, 5.18, 5.19, 5.20). This response to synthetic agonists is consistent with previously published results. For example, LaBranche, Ehrich and Eyre (2010) reported the β2-AR agonist, terbutaline, decreased iROS in BoZ activated bovine PMNs. However, they did not study resting PMNs or specifically analyze neutrophils and eosinophils. One study with resting human PMNs (Margaryan et al., 2017), also observed increased levels of CD11b expression and IL-8 secretion in response to E. This result is consistent with the current results with resting bovine neutrophils and eosinophils (Figure 5.21 and 5.23 and Table 5.3). Scanzano et al. (2015), however, analyzed both resting and activated human PMNs, as well as endogenous and synthetic adrenergic drugs. No responses were observed with resting human PMNs, in contrast to my studies with bovine PMNs and the results by Margaryan et al. (2017). Furthermore, similar to the work of LaBranche, Ehrich and Eyre (2010), E, NE and isoproterenol (β-AR agonist) were found to have a suppressive effect on

activation parameters such as ROS and CD11b expression in fMLP-activated PMNs. These results demonstrated a consistency between endogenous and synthetic AR agonists. The ROS response to E could also be reversed by propranolol, a β-AR antagonist. Propranolol, prazosin, an α1-AR antagonist, and yohimbine, an α2-AR antagonist, could also reverse E-induced suppression of CD11b expression in activated human PMNs. In contrast, CD11b expression was increased by all three synthetic AR agonists on activated bovine neutrophils and eosinophils (Table 5.3). Thus, it appears AR on activated human and bovine PMNs may mediate different responses. However, it may be difficult to directly compare these studies, as two different methods, fMLP versus BoZ + rBoIFNγ, were used to activate human and bovine PMNs. It has previously been observed with human and murine macrophages that different activation stimuli, such as LPS versus PMA, can change cell responses induced by β-AR agonists. If LPS is used to activate cells, then β-ARs mediates a suppressive effect. However, with PMA, an activator of protein kinase C, the β-ARs induced a pro-inflammatory response (Szelényi *et al.*, 2000). The observed discrepancy between iROS responses when comparing E and NE with individual synthetic AR agonists needs to be further explored to determine if this observation is unique to the culture conditions used in the current study. One possible explanation for this phenomenon could be that stimulation of multiple ARs by E and NE induces signaling that is different from signaling induced by activation of individual AR subtypes.

Most studies of PMN and macrophage responses to adrenergic agonists concluded these agonists act primarily on activated cells (Haskó *et al.*, 1995a; Gu and Seidel, 1996; Szelenyi *et al.*, 2006; Trabold, Gruber and Fröhlich, 2007; LaBranche, Ehrich and Eyre, 2010; Scanzano *et al.*, 2015). Thus, it was somewhat unexpected to observe that resting neutrophils and eosinophils displayed a range of responses with all the adrenergic agonists tested. For human PMNs, for

example, the difference between resting and activated PMNs may reflect changes in AR expression following cell activation. For example, Scanzano et al. (2015) reported resting human PMNs did not respond to adrenergic agonists. However, PMNs activated with fMLP increased AR gene expression and cell responses to adrenergic agonists were subsequently observed. Thus, it remains to be determined whether bovine PMNs truly have a different level of basal AR gene expression and function or whether the protocol I used to isolate bovine PMNs may have induced increased AR gene expression. Activation of bovine PMNs by co-stimulation with BoZ and IFNγ may also alter AR gene expression in both neutrophils and eosinophils. Further studies are required to investigate whether this activation alters expression of individual AR genes relative to resting cells and to determine whether any changes in AR gene expression correlate with the changes observed in response to adrenergic agonists.

There may be other reasons why the response of activated bovine neutrophils and eosinophils to adrenergic agonists differed relative to responses observed with resting cells (Table 5.2 and 5.3). Another factor contributing to these differences may not be changes in AR expression but rather an altered capacity of cells to respond to adrenergic agonists. Further study is required to determine if AR signaling is altered due to changes in adaptor molecules involved in signaling pathways. For example, resting neutrophils consistently "shed" L-selectin in response to all adrenergic agonists (Table 5.2) but this response was markedly decreased in activated neutrophils. BoZ + rBoIFNγ activated neutrophils have already "shed" approximately 30% of their L-selectin (Figure 5.16) and this may then reduce the cell's capacity for further L-selectin "shedding.' Similarly, activated neutrophils up-regulated iROS activity by approximately 60% (Figure 5.16). Further up-regulation of iROS in activated neutrophils and eosinophils by adrenergic agonists may be difficult to achieve if this reflects maximum cell

activity within the timeframe of the assay. Thus, there may be multiple reasons why resting and activated bovine PMNs differ in their response to adrenergic agonists. There also appear to be species differences in both the expression and function of ARs on PMNs which may also contribute to the unique responses observed with bovine PMNs.

The multiple effects of catecholamines and adrenergic drugs observed with resting PMNs isolated from bovine blood suggests adrenergic agonists can rapidly alter the phenotype of circulating neutrophils and eosinophils to a more activated form. For neutrophils, this activated form includes decreased L-selectin expression, potentially increasing the short term pool of circulating neutrophils available for pathogen surveillance and clearance (Enders *et al.*, 1995; Lynch *et al.*, 2010; Long *et al.*, 2012; Ivetic, Green and Hart, 2019; Cornell University College of Veterinary Medicine, 2020). It also includes increased neutrophil CD11b expression, increasing the ability of neutrophils to phagocytose pathogens, degranulate and respond to tissue inflammation (Berends *et al*., 1993; Tang et al. 1997; Coxon *et al.*, 1996; Ince, Weber and Scheiermann, 2019). Increased iROS in neutrophils would also increase their capacity to kill phagocytosed pathogens (Paiva and Bozza, 2014). Thus, acute stress responses with increased catecholamine release into blood could enhance host defenses in response to an invading pathogen.

Increased eosinophil CD44 expression induced by NE may have detrimental effects in allergic individuals (Katoh *et al.*, 2003). Furthermore, increased L-selectin, CD11b and CD16 expression was observed in response to catecholamines, potentially increasing eosinophil migration into tissue during a stress response and increasing eosinophils' capacity to phagocytose and respond to pathogens (Berends *et al.*, 1993; Enders *et al.*, 1995; Coxon *et al.*, 1996; Tang *et al.*, 1997; In't Veen *et al.*, 1998; Davoine *et al.*, 2002; Ince, Weber and

Scheiermann, 2019; Ivetic, Green and Hart, 2019). These functional changes may be important in ensuring cattle are better able to cope with tissue damage or infectious disease when exposed to stressful situations.

Overall, these changes in granulocytes could possibly result in phenomena such as the short-term neutrophilia observed as part of the stress leukon, as well as a stronger inflammatory response by circulating or tissue granulocytes in response to infections that may follow stressful experiences such as weaning and transportation. It is interesting to note that serum antibody responses to the opportunistic respiratory pathogens, *M. haemolytica* and *P. multocida*, also increased significantly in calves subjected to maternal separation and transportation (Malmuthuge *et al*, 2021; Manuscript submitted). Increased serum antibody responses are indicative of increased systemic exposure to these potential pathogens, but none of the calves displayed clinical signs of respiratory disease. Thus, further study of the mechanisms of stress on the adaptive immune system are also worth exploring.

6.4 Conclusions

My research provided the first complete analysis of AR gene expression in bovine leukocyte lineages and confirmed all three AR families are functional in bovine neutrophils and eosinophils. Further study of the effects of AR agonists on individual bovine leukocyte subpopulations, such as B cells and NK cells, could yield a much better understanding of how stress, acting through the SAM axis, alters a diverse range of immune functions. Differences in bovine neutrophil and eosinophil responses to AR agonists also indicate that future investigations of PMN responses to adrenergic agonists, as well as inflammatory stimuli, would be improved by analyzing separately these two PMN subpopulations. Any analysis of the effect of AR agonists, whether endogenous or synthetic, is complicated by the expression of multiple AR

genes within each PMN subpopulation and the expression of functional receptors within all three AR subtype families. Given the important role neutrophils play as a primary line of defense against infectious agents and mediator of inflammation and tissue repair, the current research suggests E and NE can modulate both host defenses and inflammation. This research also demonstrates a potentially important role for eosinophils during a stress response, with the development of an activated or allergic phenotype in response to agonists such as NE. Collectively, my studies demonstrate a potentially important role for the SAM axis in regulating the function of bovine leukocytes and many different aspects of the bovine immune system.

7.0 CONCLUSIONS AND RECOMMENDATIONS

Several general conclusions can be made from my research, as well as recommendations for improving future research in this area. Most studies reporting responses of bovine neutrophils have used PMNs. It was observed that eosinophils, depending on the individual animal and time of year, can comprise almost one third of PMN preparations. Further investigations into the impact of these temporal fluctuations in eosinophil abundance on the function of the bovine immune system should be completed.

Bovine neutrophils and eosinophils, when analyzed separately, were shown to differ in their expression of L-selectin, CD16, and CD44. Differences in the expression of these proteins may provide clues as to why neutrophils and eosinophils differ in their cell migration and responses to stimuli differently. Neutrophils and eosinophils also differed in their expression of 8 of the 9 adrenergic receptors and when activated by BoZ + rBoIFNγ responded differently to multiple adrenergic drugs. Collectively, the current data provides substantial evidence that neutrophils and eosinophils differ sufficiently in a variety of functions, and that that analyzing PMNs is not an effective approach to understanding responses to AR agonists. One factor not addressed in my *in vitro* studies was the potential that neutrophils and eosinophils may have effects on each other. Further AR agonist research with purified neutrophils and eosinophils could clarify if there is significant crosstalk between these two PMN subpopulations. It would be important to ensure, however, that the method used to purify PMN subpopulations did not alter expression or function of ARs.

Maternal separation and transportation of suckling beef calves resulted in significant changes in blood leukocyte AR gene expression. Some unique changes in AR gene expression were observed with each type of stressor indicating that how experimental animals are handled

may alter immune cell expression of AR genes. Furthermore, it was surprising that significant changes in AR gene expression were observed as much as 4 weeks after the initial stress. Therefore, a one-to-two-week adaptation period after animals are transported or moved to a new environment may not be sufficient to address these concerns. Further research is required to determine if stress alters AR gene expression in specific leukocyte subpopulations and confirm whether changes in AR transcript expression significantly leukocyte responses to AR agonists.

Based on the study of AR transcript expression in PBMC and PMN subpopulations, there are several cell populations with high AR transcript expression that could be investigated more thoroughly. Given there has been little previous study of AR activity on bovine immune cells, high AR transcript expression provides clues regarding which cell populations may be more responsive to E, NE, and other adrenergic drugs. For example, B cells were unique among all leukocyte lineages in having the highest level of α2A-AR gene expression and undetectable expression of the α2B and α2C-AR genes. Further research, using specific α2-AR agonists, such as dexmedetomidine, may reveal how the α2-AR regulates B cell activation, proliferation, and antibody production. Such studies would provide insight into the role of the α2A-AR in B cells and how stress may alter B cell function.

Bovine CD335+ ILCs, which includes both conventional NK cells and non-conventional T cells, displayed high expression of the β2, α1A and α2A-AR genes. The effect of the β2-agonist zilpaterol on CD335+ ILCs might provide insight into how this drug alters immune function when given to feedlot cattle. Some key ILC functions that may be altered by a β2-AR agonist could include cytotoxicity against virally infected cells, production of IFNγ, and the expression of CD16. For all leukocyte lineages, it may be useful to investigate the function of individual AR subtypes. Specific agonists exist to study individual β-AR subtypes and as more

α-AR subtype-specific agonists become available it will be possible to analyze the function of individual α-AR subtypes on neutrophils and eosinophils. This may provide more clarity whether individual AR subtypes mediate specific functions and their potential as therapeutic targets.

My research also provided evidence that all three AR families were expressed and functional in both neutrophils and eosinophils. Of interest, it was observed that both E and NE augmented inflammatory responses in neutrophils and eosinophils. These observations are of interest given the association between inflammation and increased neutrophil recruitment to the lung during BRD. Further analyses is now required to determine if E and NE play a direct role in this enhanced pro-inflammatory response and increased neutrophil migration and whether specific AR agonists or blockers can ameliorate these effects. It was interesting that synthetic adrenergic agonists had a strong suppressive effect on iROS, Also, both neutrophils and eosinophils increased CD11b levels in response to E, NE and synthetic agonists targeting individual AR. This increase in CD11b expression would suggest that stress can activate PMNs circulating in blood and they may have a more potent pro-inflammatory effect when recruited to the lung during BRD. Treatment with AR antagonists prior to the stress of weaning and transportation may be a novel therapeutic approach to reduce lung inflammation and the severity of BRD. Perhaps an important place to start would be to replicate the work of Rašková et al. (1987) to confirm a β-AR blocker reduces BRD morbidity and mortality with a model of controlled stress and respiratory infection. This may provide an opportunity to analyze neutrophil and eosinophil function and recruitment to infected lungs and determine if a beta-blocker significantly alters these parameters.

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